The role of nitrate reductase in the interaction between *Arabidopsis thaliana* and *Serendipita indica*

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Supervisors

Univ.Prof. Dipl.-Ing. Dr.nat.techn. Siegrid Steinkellner

Mag. Dr. Krzysztof Wieczorek

Statutory Declaration

I declare that I have authored this thesis independently, that I have not used other than the declared sources/resources, and that I have explicitly marked all material which has been quoted either literally or by content from the used sources.

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Acknowledgement

Für den gläubigen Menschen steht Gott am Anfang,

für den Wissenschaftler am Ende aller seiner Überlegungen.

Zitat von Max Planck.

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Abstract

Serendipita indica, an endophytic fungus, has the ability to enhance growth of a wide range of different host plants, including the non-mycorrhizable model plant *Arabidopsis thaliana*. It is suggested that this fungus promotes supply of nitrogen in the plants leading to increased plant growth. In the nitrate assimilation pathway, nitrate reductase (NR) is the first step-limiting enzyme. Thus, the aim of this thesis was to elucidate the role of NR and the involvement of the mitogen-activated protein kinase 6 (MPK6) during the *S. indica-A. thaliana* interaction. Therefore, the changes in transcriptional regulation of the two NR genes *Nia1* and *Nia2* triggered by the fungus were investigated. Further, different *A. thaliana* genotypes, *nia1*, *nia2*, *nia1nia2* and *mpk6*, were inoculated with *S. indica* and the growth parameters were measured. Finally, the NR assay was performed with the shoot material at different time points corresponding to different colonization phases of the fungus. This work demonstrates that only shoot weight of the wild type was significantly increased upon *S. indica* colonization. Additionally, the root morphology of all lines was changed upon fungal colonization, except for *mpk6* and *nia1nia2*. Further, at different colonization stages *S. indica* triggered changes in expression levels of *Nia1* and *Nia2*. Moreover, NR activity was increased upon *S. indica* colonization. Among all lines the *mpk6* did neither show a growth promotion nor an increase in NR activity upon fungal colonization. This study demonstrates that root colonization by *S. indica* significantly affects the NR and is associated with MPK6.

I. Introduction

Serendipita indica is an endophytic fungus that is related to the Hymenomycetes of the Basidiomycota (VARMA et al., 1999) and colonizes roots of many plant species including the model plant *Arabidopsis thaliana* (PESKAN-BERGHÖFER et al., 2004). Its colonization promotes plant growth and increases the biotic and abiotic stress resistance in plants (VARMA et al., 1999). Additionally, the application of culture filtrates containing fungal exudates as well as autoclaved cell wall extracts, lead to a plant growth promotion (VADASSERY et al., 2009). The colonization is limited to the roots and offers *S. indica* as a new tool for the improvement of plant production systems. The ability of the fungus to grow axenically makes it very interesting for research and therefore it becomes a model organism (VARMA et al., 1999). Since *S. indica* was first discovered at the end of the 19th century, many mechanisms of action are largely unknown including the nitrogen assimilation pathway in plants during fungal colonization. A higher nitrogen uptake increases plant growth and further leads to a higher yield in plants (CRAWFORD 1995). Perhaps the nitrate pollution in the groundwater caused by excessive N fertilizer could also be reduced due to increased N uptake. Therefore a proof of increased nitrate uptake promoted by the fungus would be necessary. In this work, the relationship between N uptake, plant growth and the genes important for nitrate reductase is elucidated during *S. indica* colonization.

1. Aim of this work

The aim of this work was to test whether there is a relation between increased nitrate uptake performed by plants, *Serendipita indica* root colonization and growth promotion triggered by the fungus. The broad host range of *S. indica* and its ability to grow axenically has been exploited to use the model plant *Arabidopsis thaliana* in this work. Therefore, different *A. thaliana* genotypes were grown on Knop medium to show the influence of nitrate reductase and MPK6 on plant growth upon fungal colonization. To investigate the onset of growth promotion triggered by the fungus, three interesting colonization steps are considered: 3 dpi (biotrophic phase), 7 dpi (cell death associated phase) and 14 dpi (fungal reproduction phase).

2. Hypotheses

Hypothesis 1: Does *S. indica* change the expression of *Nia1* and *Nia2* genes in colonized plants?

Hypothesis 2: Does *S. indica* colonize all used genotypes to similar extend?

Hypothesis 2: Does the *S. indica* root colonization trigger changes in the biomass as well as in root morphology of the respective genotypes?

Hypothesis 3: Does *S. indica* influence the activity of nitrate reductase in the different genotypes?

3. Literature review

3.1. Organisms

3.1.1. *Arabidopsis thaliana*

Arabidopsis thaliana is a commonly used model plant due to its short generation time, self-pollination and small genome, which is completely sequenced. Therefore, *A. thaliana* represents an essential tool for understanding molecular mechanisms. Moreover, the availability of different mutants and the fact that the fungus *Serendipita indica* is able to colonize *A. thaliana* were essential prerequisites for this study (PESKAN-BERGHÖFER et al., 2004).

3.1.2. The root endophyte *Serendipita indica*

The endophytic fungus *S. indica* belongs to the *Sebacinaceae* family and was first isolated in 1997 in the desert of Rajasthan in northwest India. It is a filamentous fungus, which forms pear-shaped thick-walled chlamydospores at the end of thin-walled hyphae (VERMA et al., 1998). A first contact between *S. indica* hyphae and plant roots leads to a development of appressoria and to inter- and intracellular colonization of rhizodermal, cortical and root hair cells. *S. indica* never invades stellar tissue or the plant shoots (VARMA et al., 1998). At seven days after inoculation with the fungus an external sporulation and further intracellular colonization takes place. In addition, colonized root cells die. After 14 days when the fungus colonizes cortical cells intracellularly it forms coils or round bodies and branches. The round bodies might function as a storage organ but no arbuscule, which are characteristic for arbuscular mycorrhizal fungi (AMF), is formed. It has been reported that *S. indica* behave similarly to typical AMF but it can colonize roots of a wide host range and can be cultivated axenically. *S. indica* has the ability to promote plant growth and enhances resistance to biotic and abiotic stresses (PESKAN-BERGHÖFER et al., 2004) significantly influencing the plant innate immune system (SCHÄFER and KOGEL, 2009). In addition, the plant phosphate and nitrate uptake is increased. The growth

promotion effects triggered by *S. indica* involve a co-regulated stimulation of enzymes in the nitrate and starch metabolism (SHERAMETI et al., 2005). However, the genome of *S. indica* does not contain a nitrate transporter gene (LAHRMANN et al., 2015). Consequently, *S. indica* grows considerably slower when supplied only with nitrate (LAHRMANN et al., 2013).

3.2. Nitrate assimilation by *Arabidopsis thaliana*

Nitrate is the most common inorganic source of nitrogen used by plants. Its availability in soil is frequently the most limiting factor influencing their growth. Nitrogen is recruited by plants either as nitrate or ammonium or via nitrogen fixation with the help of rhizobia (SHERAMETI et al., 2005).

Environmental nitrate has many effects on plants, which include the nitrate transport system, changes in root morphology, increased root respiration and especially the nitrate and nitrite reductase activity (REDINBAUGH and CAMPBELL, 1991).

Nitrate is usually taken up via a proton gradient by the roots across the plasma membrane of epidermal and cortical cells but can be also efficiently absorbed by leaf cells (CRAWFORD and GLASS, 1998). Two gene families *AtNRT1* and *AtNRT2* are involved in the nitrate uptake. Genes of the *NRT1* family represent the low affinity transport system (LATS), whereas *NRT2* genes code for the high affinity transport system (HATS). Each of these systems is nitrate inducible and consists of a constitutive component (DE ANGELI et al., 2009).

Once nitrate is in the plant cell it can be: (1) effluxed back into the apoplasm, (2) influxed across the tonoplast membrane into the vacuole, (3) further distributed by xylem for long-distance translocation to the leaves (CRAWFORD and GLASS, 1998), and (4) finally reduced to nitrite. Nitrate reduction as energy consuming process takes place in all plant parts in the cytoplasm and is catalyzed by the nitrate reductase (NR) with NADH or NADPH serving as reductant. In *A. thaliana Nia1* and *Nia2* genes encode the NR, which reduces nitrate to toxic nitrite.

In contrast to nitrate, that can be stored in vacuoles, nitrite is not accumulated in plants in high levels (CRAWFORD 1995). Ammonia is synthesized from nitrite in shoots,

which is driven by nitrite reductase in chloroplasts with ferredoxin as reductant (reviewed BEEVERS and HAGEMANN, 1969). In roots, nitrite reductase activity occurs in leucoplasts (HELDT and PIECHULLA, 2015), where nitrite is fixed in glutamate and further metabolized in glutamine (CRAWFORD 1995). However, also glutamate can be formed out of glutamine and 2-oxoglutarate by glutamate synthase (see **Figure 1**Figure 1, LUO et al., 2013). Glutamine is an amino acid that is used in the biosynthesis of many proteins (LEA and MIFLIN, 2003).

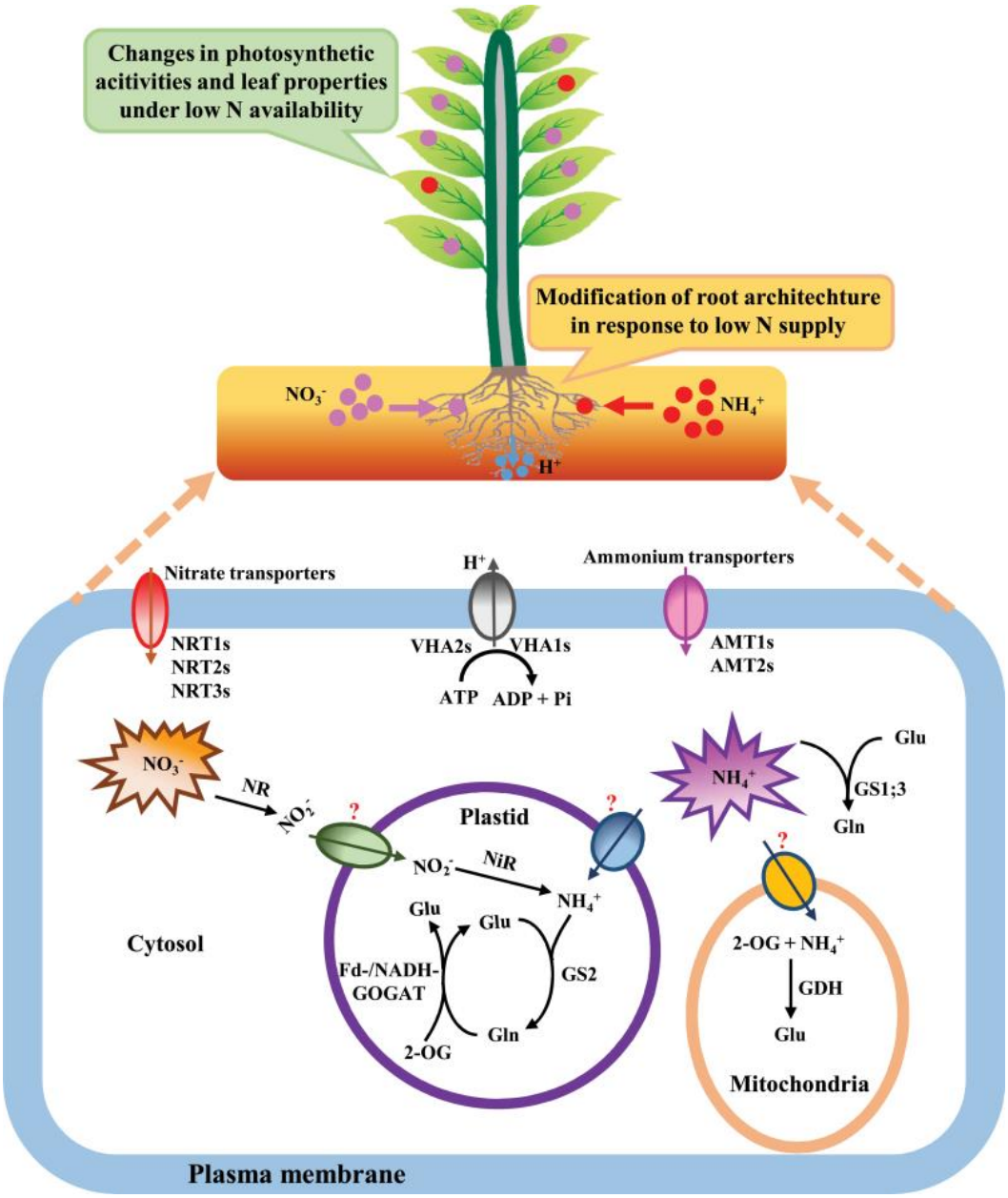

Figure 1 A concept of the nitrogen metabolism in plants. In the roots Nitrate (NO3-) and ammonium (NH4+) enter the cytosol with the help of nitrate transporters (NRT) and ammonium transporters (AMT), respectively, simultaneously with H+ATPases (VHAs). Then a NO3- translocation to upper plant parts can take place. The cytosolic nitrate reductase (NR) and the chloroplastic/plastidic nitrite reductase (NiR) convert NO3- to NH4+. Subsequently, NH4+ can be assimilated to glutamine (Gln) catalysed by glutamine synthetase (GS) isoenzymes either in the cytosol or the plastid. Further, the Gln in the plastid with 2-oxoglutarate (2-OG) can be metabolized to glutamate (Glu) by ferredoxin (Fd)- or NADH-dependent glutamate synthase (Fd/NADH-GOGAT). In addition, NH4+ can be converted to Glu by glutamate dehydrogenase (GDH) in the mitochondrion (LUO et al., 2013).

3.3. Nitrate reductase

3.3.1. Transcriptional regulation of NR

In *A. thaliana* two genes *Nia1* and *Nia2* encode the NR. *Nia2* is responsible for 90 % of the NR activity in wild type plants and *Nia1* for the remaining 10 % (WILKINSON and CRAWFORD, 1991). The major gene *Nia2* is expressed mainly in roots rather than leaves (CHENG et al., 1991). However, plants in which the entire *Nia2* gene is missing, grow normally with nitrate as sole nitrogen source (WILKINSON and CRAWFORD, 1993). In *A. thaliana* wild type seedlings grown on a cytokinin-rich medium an increased NR activity could be observed as a result of the increased *Nia1* expression. Therefore, a different regulation of both *Nia* genes can be assumed (YU et al., 1998). Their expression is induced by nitrate and repressed by downstream metabolites such as ammonia or glutamine formed out of nitrate (HOFF et al., 1994). At the end of the night the amount of NR mRNA reaches a maximum in nitrate-replete plants and decreases rapidly during the photoperiod. The diurnal changes in NR mRNA levels occur due to the accumulation of glutamine during the photoperiod (SCHEIBLE et al., 1997).

3.3.2. Post-translational regulation of NR

Post-translational regulation of NR is very important to avoid accumulation of toxic nitrite. Accordingly, NR in higher plants is strongly influenced by environmental stimuli and various treatments. A decrease in carbon dioxide and a loss of light lead to an inactivation of NR. Sugars and sugar phosphates are the major internal triggers for the rapid activation/inactivation of the NR (KAISER and HUBER, 2001; LILLO et al., 2004).

Three different states of NR occur: (1) free active, (2) phosphorylated active and (3) inactive with an additional 14-3-3 inhibitor protein (KAISER and HUBER, 2001).

Phosphate buffers trigger high NR activity through binding divalent cations that are responsible for NR modulation. Total NR activity can be measured in the presence of excess EDTA and the active form with potassium (KAISER and HUBER, 2001).

3.3.3. Distribution, synthesis and function

NR is the first step-limiting enzyme in the nitrate assimilation pathway in higher plants (BEEVERS and HAGEMANN, 1969) and it is primarily located in root epidermal and cortical cells as well as in shoots in the mesophyll cells (SIDDIQI and GLASS, 2002). Following characteristics for the enzyme have been previously reported: (1) the molecular weight is between 500 and 600 kDa; (2) it contains Mo and FAD (reviewed BEEVERS and HAGEMANN, 1969); (3) When NADH is the electron donator the optimal pH is 7 to 8 and when NADPH the optimal pH is 6 to 6.25, respectively (CAMPELL 1999); (4) maximum enzyme activity of 600 to 700 µmoles nitrite per minute and mg protein has been measured in spinach; (5) it underlies a diurnal rhythm and is very unstable both *in vitro* and *in vivo* (reviewed BEEVERS and HAGEMANN, 1969).

NR has received a lot of experimental attention due to its complex regulation system. First, NR expression and activity are mainly influenced by the light conditions and nitrate concentration, although phytohormones, N- and C-metabolites play an effective role as well. The enzyme regulation occurs at transcriptional and translational level as well as via phosphorylation during post-translational modifications in response to environmental factors (YANAGISAWA 2014). In addition NR activity rapidly in- or decreases by a variety of endogenous promoters and inhibitors (MACKINTOSH et al., 1995). Reversible phosphorylation allows very rapid regulation of NR activity (KAISER and HUBER, 2001).

NR catalyzes the reduction from nitrate to toxic nitrite in the cytoplasm of plant cells. A secondary function of NR is catalyzing the nitric oxide (NO) from nitrite and molecular oxygen to superoxide (YAMASAKI and SAKIHAMA, 2000; ROCKEL et al., 2002). Pathogens induce expression of the NR genes (YAMAMOTO et al., 2003), which results in higher NO accumulation in *A. thaliana* cells inducing cell death (CLARKE et al., 2000).

3.4. Interaction between MPK6 and nitrate reductase

In *A. thaliana*, MPK6 is a well-characterized mitogen-activated protein kinase (MAPK) that transduces environmental stimuli into intracellular responses via MAPK cascades. Up-streamed MAPK kinases (MAPKKs) activate MAPK by phosphorylation, which are activated by MAPKK kinases. MAPK cascades are activated by various biotic and abiotic factors (ICHIMURA et al., 2000). MPK6 has many functions in plants including the innate immunity, environmental stress responses, plant hormones signaling and a novel function in plant growth and development (ZHANG and KLESSIG, 2001; LIU and ZANG, 2004; FEILNER et al., 2005).

A direct interaction between MPK6 and NR is identified both *in vivo* and *in vitro*. In *A. thaliana* phosphorylation of NIA2 by MPK6 increases NR activity and NO production and changes the root morphology. The minor NIA1 protein is not phosphorylated by MPK6 (WANG et al., 2010).

II. Materials and Methods

1. Organisms

Experiments were conducted on wild type *Arabidopsis thaliana* Columbia (Col-0) and on the following mutant lines: *nia1* (N504164; T-DNA insertion; in background Col-0), *nia2* (N575996, T-DNA insertion, in background Col-0), *nia1nia2* (CS2356; Ploidy 2: nia2-5 knockout and nia1-1 single nucleotide substitution A to T; in background Col-0) and *mpk6* (CS31099; T-DNA insertion, in background Col-0). The seeds of *A. thaliana* Col-0 were maintained in the lab. The lines *nia1* and *nia2* were ordered from TAIR (https://www.arabidopsis.org/) and provided by ABRC (Biological Arabidopsis Resource Center (https://abrc.osu.edu/order-stocks). Seeds of *mpk6* were kindly provided by Dr. Jürgen Klein-Vehn (Department of Applied Genetics and Cell Biology, Division of Plant Genetics and Cell Biology, University of Natural Resources and Life Sciences, Vienna) and *nia1nia2* seeds by Prof. Thorsten Hamann (Department of Biology, Norwegian University of Science and Technology, Trondheim, Norway). The fungus *S. indica* (see **Figure 2**) was kindly provided by Prof. Ralf Oelmüller (Department of General Botany and Plant physiology, Biological-Pharmaceutical Faculty, Friedrich-Schiller-University, Jena, Germany) and the strain preservation took place on the Kaefer medium.

Figure 2 *S. indica* on Kaefer medium after 20 days

2. Fungal cultivation

In order to obtain KAEFER medium (HILL and KAEFER, 2001 according to JOHNSON et al., 2011) for the *S. indica* inoculum, stock solutions were first prepared. For the macronutrient mix stock solution, 12 g of $NaNO_3$, 10.4 g of KCl, 10.4 g of $MgSO_4*7H_2O$ and 30.4 g of KH_2PO_4 were dissolved in 1 liter of distilled water and then autoclaved (20 min, 121 ° C., 1.2 bar). The micronutrient mix stock solution was prepared out of 2.2 g of $ZnSO_4*7H_2O$, 0.16 g of $CuSO_4*5H_2O$, 0.5 g of $MnSO_4*4H_2O$, 0.11 g (NH_4) $6Mo7O_{24}*4H_2O$, 0.16 g of $CoCl_2*$ $5H_2O$ and 1.1 g of H_3BO_3. Then the micronutrient solution was dissolved in 1 liter of distilled water through stirring using a magnetic stirrer and afterwards autoclaved. For the Fe-EDTA stock culture, 2.5 g of $FeSO_4*7H_2O$ was dissolved in 400 ml of distilled water. Thereafter, 3.36 g of $Na_2EDTA*2H_2O$ was added and the solution was boiled in a microwave. The mixture was then stirred for about 30 minutes until the solution had cooled and the volume was adjusted to 450 ml. All stock cultures were stored at 4 ° C. After preparation, 0.1 g of thiamine, 0.01 g of nicotinic acid 0.01 g of pyridoxine and 0.04 g of glycine were dissolved in 100 ml of sterile water and then filter-sterilized. Afterwards the stock solution was stored in portions of 1 ml in 1.5 ml Eppendorf tubes at -20 ° C. Kaefer medium preparation was done in 1 liter Erlenmeyer flasks (see **Table 7** in the appendix). For one liter, 20 g of D-glucose, 2 g of peptone, 1 g of yeast extract and 1 g of casein hydrolysate were weighed. Then, 50 ml of the macronutrients mixture, 10 ml of the micronutrient mixture and 1 ml of Fe-EDTA were added. Subsequently, the pH was adjusted to 6.5 with 10 N KOH and afterwards the solution was autoclaved. As soon as the solution had about 55 °C, 1 ml of a sterile vitamin mix solution was added under the laminar flow hood and stirred before pouring it to Petri dishes (9 cm). After the medium had solidified, a 0.7 mm Kaefer medium disc containing *S. indica* was placed in the middle of the Petri dishes filled with Kaefer medium. The disc surface with mycelium was placed on the top of the medium and was closed with Parafilm. After 20 days (dark and at room temperature) the culture was used to inoculate the plants.

3. Experimental setup

The sterilization of all *A. thaliana* seeds was performed under sterile conditions in a laminar flow hood. Seeds were sterilized for 8 minutes in a 2 ml Eppendorf tube with a sterilization solution containing 10 ml sterile ddH_2O, 12.5 ml EtOH (70 %), 2.5 ml DanKlorix and two drops of Tween 20. Subsequently, seeds were washed three times for one minute with 1 ml sterile ddH_2O. Finally they were transferred using a pipette onto a sterile filter paper for drying. If the seeds were not used immediately, the Petri dishes were closed with Parafilm and then stored under dark conditions at 4 °C.

For pre-checking the mutant lines *nia1* and *nia2*, MS medium (MURASHIGE and SKOOG, 1962) was chosen because it is simple to produce and enables good plant growth. To prepare 1 liter of medium a 1 liter Erlenmeyer flask was used. 20 g sucrose, 2.3 g MS salts and 7 g Daishin agar were dissolved in 1 liter of distilled water and the pH was adjusted to 5.9 with 1 M KOH while stirring. Then the medium was autoclaved and after cooling down 0.5 ml sterile filtered kanamycin was added under the laminar flow hood. Subsequently, the liquid medium was poured into the 9 cm Petri dishes and was allowed to solidify. Afterwards, 80 to 100 dry seeds were sprinkled onto the medium per Petri dish. Plates were incubated at 23 °C with a photoperiod of 16 h light and 8 h dark in a climate controlled cabinet. The positively selected transgenic plants were used for PCR to look if the inserted T-DNA is existing.

All other experiments were carried out using the 0.2 Knop medium (KNOP 1865 according to SIJMONS et al., 1991). Therefore five stock solutions were prepared. The respective chemicals (see **Table 1**) were dissolved in 1 liter distilled water and afterwards autoclaved (20 min, 121 °C, and 1.2 bar). All stock solutions were stored at 4 °C. For 1 liter medium 20 g glucose and 8 g Daishin agar were weighed in a 1 liter Erlenmeyer flask including a magnetic stirrer and filled up with 1 liter distilled water. Then, 2 ml of the stock solutions I, II and III, 0.4 ml of the stock solution IV and 0.2 ml of the stock solution V were added. Subsequently, the pH was adjusted to 6.4 with 1 M KOH under the usage of the magnetic stirrer. Afterwards the solution

was autoclaved and during that time the frozen B5 vitamin solution was melted. As soon as the solution had about 50 °C, 1 ml of the filter sterilized B5 vitamin solution was added under the laminar flow hood and stirred with the magnetic stirrer.

Table 1 Stock solutions for Knop medium per liter (KNOP 1865)

Stock solution	Chemical	g per liter
I	KNO_3	121.32 g
	$MgSO_4 \cdot 7H_2O$	19.71 g
II	$Ca(NO_3)_2 \cdot 4H_2O$	120 g
III	KH_2PO_4	27.22 g
IV	FeNaEDTA	7.34 g
V	H_3BO_3	2,86 g
	$MnCl_2$	1.81 g
	$CuSO_4 \cdot 5H_2O$	0.073 g
	$ZnSO_4 \cdot 7H_2O$	0.36 g
	$CoCl_2 \cdot 6H_2O$	0.03 g
	H_2MoO_4	0.052 g
	NaCl	2 g

For all experiments the medium was poured into 9 cm Petri dishes except for NR activity assay where it was filled into 145 x 20 mm Petri dishes with vents and was allowed to solidify. To get enough plant material bigger Petri dishes were used for the NR activity assay. Afterwards, the respective prior sterilized seeds were put onto the medium using a tweezer (see **Figure 3)**. At later time points for root length/ branching less plants were used within one Petri dish otherwise no exact root harvest would have been possible.

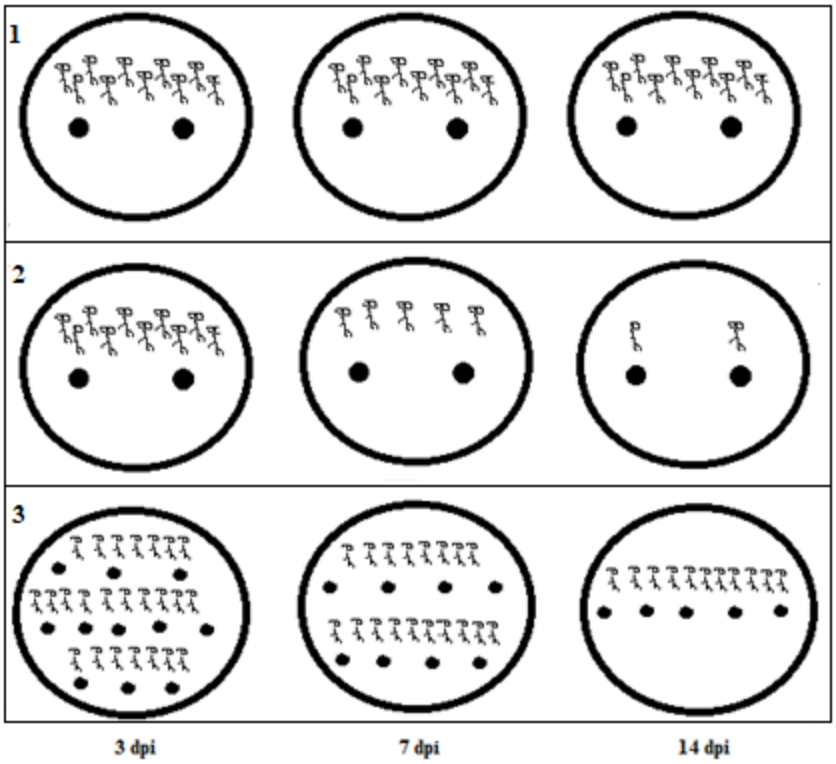

Figure 3 Schematic sowing plan of *A. thaliana* seeds with *S. indica* inoculation at 3, 7 and 14 dpi on Knop medium. 1: for fresh/dry weight, colonization rate, gene expression; 2: for root length/ branching; 3: for NR activity assay.

Then the plates were placed in a climate chamber with 16 h light and 8 h darkness at 23°C. After 5 days Petri dishes were arranged in a 45° position to stimulate a downward root growth (see **Figure 4**).

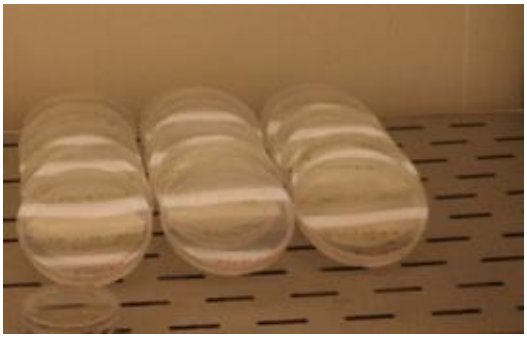

Figure 4 Position of Petri dishes after 5 days in a climate chamber.

After ten days *A. thaliana* plants were inoculated (see **Figure 3**) with 0.7 mm Kaefer medium discs containing *S. indica* and Kaefer medium discs as control. The disc

surface with mycelium was placed on the top of the medium at a distance of 0.5 cm from the roots using a sterile tweezer. All shoot and root samples were harvested 3, 7 or 14 days after inoculation. They were immediately shock frozen in liquid nitrogen and grounded using mortar and pestle or the mill. Not immediately used samples were stored under dark conditions at -80 °C.

For seed production (see **Figure 5**), positively selected plate-grown transgenic plants were transferred to autoclaved soil (Gramoflor Kultursubstrat, Substratgruppe 1) and cultivated at 25 °C with a 16 h light and 8 h dark period in a climate controlled cabinet. As required plants were watered with tap water.

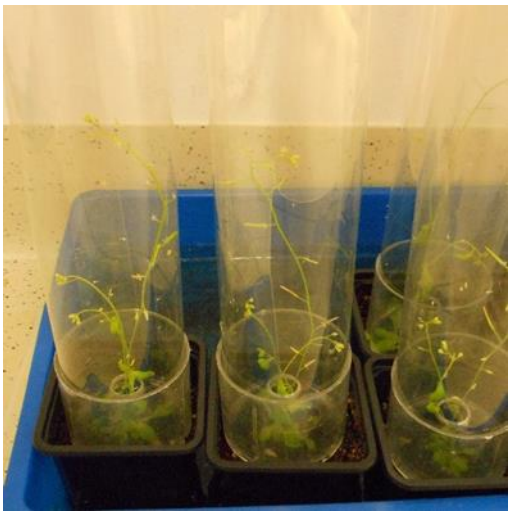

Figure 5 *A. thaliana* cultivated for seed production.

3.1. Selection of transgenic plants

For a pre-selection the mutant lines *nia1* and *nia2* were sprinkled onto the MS medium containing kanamycin to look which plants were able to grow on it because these two transgenic lines should have possessed a kanamycin resistance gene. After 14 days it worked perfectly for *nia1* but not for *nia2*. Most probably the kanamycin resistance gene was silenced in this line, so all of them died. For that reason ten seeds of the line *nia2* were seeded on MS medium without kanamycin again. Then, 80 mg leaf tissue of ten individual 20-day-old *nia2* and prior selected *nia1* seedlings were sampled for DNA extraction. Before the selection of the homozygous lines *nia1* and *nia2* can begin, single mutants specific primer pairs had to be tested with a PCR using gDNA from *A. thaliana* Col-0 as template. Isolation of genomic DNA for PCR screening was performed with Qiagen DNeasy Plant Mini Kit. 80 mg of plant leaves were ground in liquid nitrogen and transferred into a 1.5 ml Eppendorf tube. DNA isolation procedure was performed according to the manufacturer's instructions. DNA concentration was measured with NanoDrop 2000c. Then a polymerase chain reaction was performed to amplify DNA fragments in a master cycler. The PCR reaction was conducted as follows: 94 °C for 5 min, then 30 cycles of 94 °C for 20 s, 58 °C for 30 s and 72 °C for 30 s, at the end 72 °C for 8 min. It was used to find homozygous individuals for following Salk lines: for *nia1* mutant (line Salk_004164): nia1_Salk_004164_for and nia1_Salk_004164_rev; and for *nia2* mutant (line Salk_088070): nia2_Salk088070_for and nia2_Salk088070_rev, respectively (for primer sequences see **Table 2**). For one reaction 0.5 µl gDNA was used in 2 µl 10x PCR buffer (Taq polymerase), 1 µl dNTPs (10 µM), 0.5 µl forward primer (10 µM), 0.5 µl reverse primer (10µM) and 0.2 µl Taq polymerase and MiliQ-water to reach the final volume of 20 µl. Milli Q water is ultra-pure water under whose use a perfect reproducibility and accuracy in the ppm range can be achieved. Two types of master mix were prepared, one comprising the T-DNA border primer and the gene-specific primer. The second master mix included a gene-specific primer pair that amplifies the wild type DNA.

Table 2 List of primers for genotyping T-DNA insertional mutants

Line	Name of Primer	Primer sequence 5'- 3'
T-DNA specific primer used for all lines	LBa1 (ALONSO et al., 2003)	TGGTTCACGTAGTGGGCCATCG
	LBb1 (ALONSO et al., 2003)	GCGTGGACCGCTTGCTGCAACT
SALK_ 004164	nia1_Salk_004164for ALONSO et al., 2003)	CGTTAAACGTTTGCTTCGAAG
	nia1_Salk_004164rev (ALONSO et al., 2003)	TAATCTACATAATTGCCCCGC
SALK_ 088070	nia2_Salk_008070for (ALONSO et al., 2003)	CTTTGGTAGACGCCGAACTC
	nia2_Salk_008070rev (ALONSO et al., 2003)	TTGGCTTTATCCGAGTGAATG

After PCR, a gel electrophoresis was carried out to make the single PCR products visible. DNA separation was done using 1 g agarose in 100 ml 1x TAE Buffer (40 mM TRIS-HCl, 40 mM glacial acidic acid, 1mM Na-EDTA) containing 4 µl peqGreen. 20 µl PCR product was mixed with 4 µl Loading Buffer before loading on the gel. 6 µl Ladder (100 bp) was used. The intensity of the voltage was 90 V provided by a PowerPac 200 supply system and the electrophoresis was run until the dye front reached the last third of the gel. The separated DNA fragments were visualized through UV light and documented via 245 nm GelDoc XR+ system supported by a LabTM Software version 4.0.1. Selected primers amplified specifically single products of expected sizes, 1186 bp for *Nia1* and 556 bp for *Nia2*, respectively. **Figure 6** shows the electrophoresis gels with obtained results.

Figure 6 Test of primer pair for *Nia1* and *Nia2* at 58°C. a) *Nia1*; product size: 1186 bp. b) *Nia2*; product size: 556 bp. c) comparison between *Nia1* and *Nia2* amplicons.

After the primer test was successful the existence of the T-DNA insertion in the two mutant lines was examined for each individual plant. Following picture describes how to identify homozygous lines (see **Figure 7**). Subsequently, homozygous plant lines were used for propagation on soil to get enough seeds for further experiments.

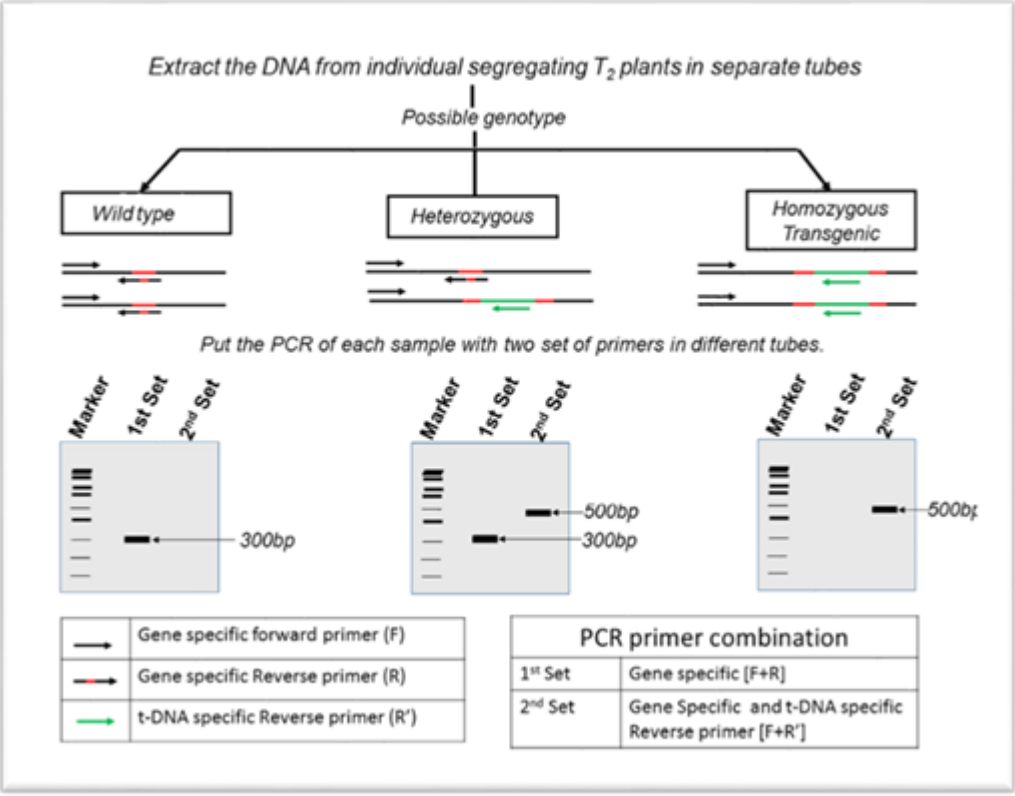

Figure 7 Selection of transgenic plants. This figure shows the primer combinations and the following PCR products to identify T-DNA insertion mutants. The red color shows the site of insertion for the T-DNA, green is representing the inserted T-DNA sequence. Using a wild type (no insertion mutation), a smaller PCR product (300 bp) appears on the agarose gel. If the plant is hemizygous then the PCA amplification shows two differently sized bands and for the homozygous line a single larger band (500 bp) appears (PASSRICHA et al., 2016). In this work *Nia1* and *Nia2* primer pair was used as a gene specific primer. LBb1 and LBa1 as T-DNA specific reverse primer.

3.2. Gene expression analysis

Per treatment, an amount of approximately 80 mg plant material was weighed and ground in liquid nitrogen using mortar and pistil. The fine powder was transferred into a 2 ml Eppendorf tube. The plant material, which was not immediately used for further experiments, was stored at -80°C. Total RNA was isolated from shoots and roots using

the Qiagen RNeasy Mini Kit. To remove DNA traces additional digestion was done using Qiagen RNase-free DNAse Set. All steps were performed according to the manufacturer's instructions. Purity and quantity of total RNA was tested using NanoDrop 2000c. The absorbance measurements for RNA concentration were determined at 260 nm. For pure RNA the ratio of absorbance at 260 and 280 nm should be a value of 2. In addition, the wild type RNA samples were tested with 2100 Agilent bioanalyzer. All required steps were performed according to the manufacturer's instructions. RNA quality was determined with the help of the RNA Integrity Number (RIN) using the entire electrophoretic trace (see **Figure 8**). All samples with a RIN more than 8 were used for cDNA synthesis.

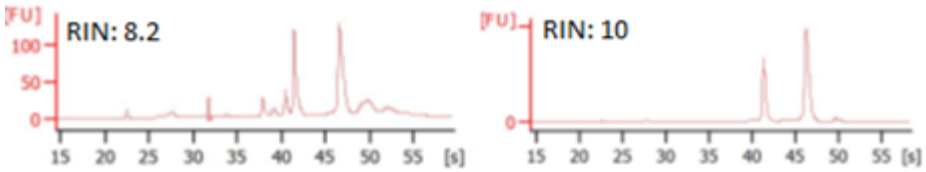

Figure 8 Electropherogram of total RNA Integrity Number (RIN). A RIN of 10 indicates a good quality of total RNA.

cDNA samples were prepared using the reverse transcriptase kit SuperScript III First-Strand System. One RT-PCR mix containing 11 µl template RNA, 2 µl reverse primer (N6 random primer 10 µM) and 1 µl dNTPs (10 mM) was incubated for 5 minutes at 65°C in a thermocycler followed by incubation on ice for at least 1 minute. Afterwards, 4 µl 5x FS-Buffer, 1 µl DTT (100 mM), 0.5 µl RNase Inhibitor and 0.5 µl Superscript Reverse Transcriptase were added to the RT-PCR mix. Subsequently, reaction mix was incubated in a master cycler. The program was conducted as follows: 25 °C for 5 min, 50 °C for 60 min and 70 °C for 15 min. cDNA was stored at 4 °C for a short time and for longer periods at -20°C. To analyze changes in the expression of *Nia1* and *Nia2* with quantitative real-time PCR (qPCR) primer pairs for *Nia1* (Nia1_RTfor and Nia1_RTrev) and *Nia2* (Nia2_RTfor and Nia2_RTrev) were tested for their specificity (for primer sequences see **Table 3**).

Table 3 Primer sequences (Sigma-Aldrich, Austria) used for quantitative real-time PCR

	Primer sequence 5'- 3'	Tm°
Nia1_RTfor (WIECZOREK 2016)	ATCGTCAAAGAAACCGAAGTC	62,0
Nia1_RTrev (WIECZOREK 2016)	ACGGAGCATGGATGAGTT	60,7
Nia2_RTfor (WIECZOREK 2016)	GGTTACGCATATTCCGGAG	61,9
Nia2_RTrev (WIECZOREK 2016)	CATGCACGAACAGCAATC	61,6
UBP22for (HOFMANN et al., 2007)	CAATGCAGCAACGCCAGA	61,9
UBP22rev (HOFMANN et al., 2007)	AGCAGCAGTAGCACCACCAC	61,9

Each of the primer pairs designed for qPCR amplified specifically single product of expected size, 210 bp for *Nia1* and 200 bp for *Nia2*, respectively. **Figure 9** shows the electrophoresis gels with obtained results.

Figure 9 Test of primer pairs for qPCR. a) Primer pair *Nia1*_RT tested for *Nia1* expression analysis, product size 210 bp; b) Primer pair *Nia2*_RT tested for *Nia2* expression analysis, product size 200 bp.

Quantitative real-time PCR of At1g77760 (*Nia1*) and At1g37130 (*Nia2*) expression in roots and shoots was performed using a PeqStar 96Q Light Cycler. Each sample contained 2 µl cDNA, 10 µl Kappa SYBR Green Universal qPCR Mix, 0.5 µl forward primer (10 µM), 0.5 µl reverse primer (10 µM) and ddH_20 to reach the final volume 20 µl (WIECZOREK and BOHLMANN, 2016). *UBP22* was used as an internal reference gene and the calculation of the fold changes was done according to the $2^{-\Delta\Delta Ct}$ method (LIVAK and SCHMITTGEN, 2001). The primer sequences are shown in **Table 3**.

3.3. Colonization rate of *S. indica*

To check the presence of fungal DNA within the plant roots 80 mg roots were first sampled and ground under liquid nitrogen, transferred into a 2 ml Eppendorf tube and if not used immediately, stored at -80°C. Otherwise DNA extraction was done with Qiagen DNeasy Plant Mini Kit. DNA isolation procedure was performed according to the manufacturer's instructions. DNA concentration was measured with NanoDrop 2000c. Subsequently, the colonization rate was determined by quantitative real-time PCR using *S. indica*-specific PiTEF gene and *AtUBQ5* as an internal reference plant gene. Each sample contained 2 µl gDNA, 10 µl Kappa SYBR Green Universal qPCR Mix, 0.5 µl forward primer (10 µM), 0.5 µl reverse primer (10 µM) and ddH_20 to reach the final volume 20 µl (WIECZOREK and BOHLMANN, 2016). The fungal colonization was calculated by the ΔCt method (LIVAK and SCHMITTGEN, 2001). For primer sequences see the **Table 4**.

Table 4 Primer sequences (Sigma-Aldrich, Austria) used for the colonization rate

Name	Primer sequence 5'- 3'
PiTEF1for (DANESHKHAH et al., 2013)	ACCGTCTTGG GGTTGTATCC
PiTEF1rev (DANESHKHAH et al., 2013)	TCGTCGGTGTCAACAAGATG
AtUBQ5for (DANESHKHAH et al., 2013)	CCAAGCCGAAGAAGATCAAG
AtUBQ5rev (DANESHKHAH et al., 2013)	ATGACTCGCCATGAAAGTCC

3.4. Growth parameters

The effects of *S. indica* on the growth parameters of different *A. thaliana* lines were investigated by fresh and dry weight as well as root length and root branching measurements. For one repetition ten seeds of the respective line (Col-0, *mpk6*, *nia1*, *nia2*, *nia1nia2*) were cultivated on Knop medium (see **Figure 3**). The inoculation of *S. indica* was done as described above.

To measure the fresh weight shoots were cut off from the roots and weighed separately in small plastic bowls (see **Figure 10**). Then the root and shoot material were put in a drying oven at 70°C.

After ten hours the dry weight was measured using a new plastic bowl because its weight changed during the drying process.

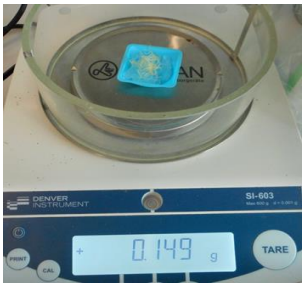

Figure 10 Root sample used to measure fresh weight

To determine the root length each individual plant was carefully harvested by pulping the agar medium. Subsequently the roots were pulled from the pulped medium and put on a paper towel to remove remaining agar. Then the elongated primary root was measured with a ruler. The root branches were counted with the aid of a light microscope (Olympus SZX2-ILLT) for 3 and 7 dpi. For 14 dpi the WinRHIZO Pro was used. This system produce digital pictures and analyses root architecture, morphology and topology. A root sample was placed in a water-filled transparent dish and scanned.

3.5 Nitrate reductase activity assay

The nitrate reductase activity assay is a colorimetric enzymatic assay for the determination of nitrite in plant shoots and roots. The synthesized nitrite is measured colorimetrically at 540 nm using sulfanilamide (SA) and N-(1-Naphthyl) ethylene diamine dihydrochloride (NED). The principle can be described as follows: NO_3^- in combination with ß-NADH NR results in NO_2^-, ß-NAD and H_2O. After SA addition, a diazonium salt is formed. This in mixture with NED produces a nitrite color complex, which colors the sample magenta (SIGMA-ALDRICH 1997).

In this work it is performed *in vitro* as a spectrophotometric stop rate determination. Before starting with the samples a proof of concept was done by covering 50 plants (ten per petri dish) with 10 mM $NaNO_3$ or with ddH_2O as control treatment. The plates were placed under light for 3.5 h at 21 °C, then harvested and subsequently treated as described below.

300 mg of ground shoot and root material (sometimes less root material was harvested) was transferred into a 2 ml Eppendorf tube and frozen at -80 °C. During thawing of the powder, ice cold extraction buffer (pH 8) containing 1 M TRIS-HCl (pH 8), 0.1 M EDTA, 0.01 M $Na_2MoO_4*2H_2O$, 5 mM FAD, 0.1 M DTT, 10 % BSA, 14.3 M EtOH and 0.1 M PMSF was added. For shoots a relation of 1:5 (w/v) and for roots a relation of 1:2 (w/v) was used. Shoot samples were centrifuged at 13000 rpm at 4 °C for 5 min and roots were centrifuged three times for 5 min at the same conditions using a 5415R Centrifuge. The supernatant was collected and transferred into a new precooled tube. Subsequently, supernatant was added to the reaction buffer already being in the microtiter plate (Greiner CELLSTAR®96). The reaction buffer contained 1 M $NaNO_3$, 0.5 M $Na_2HPO_4*12H_20$ and 0.1 M $NaH_2PO_4*2H_20$ at pH 7.5. 5 mM NADH was then added to half of the samples in order to start the reaction. Shoot samples were incubated in dark at 30 °C for 30 min and the root samples for 60 min in an incubator. Nitrite determination was done by adding 10 % SA in 2 M HCl to stop the reaction followed by 1 % NED. The samples were mixed thoroughly. The color was allowed to develop for 10 min at room temperature and protected from light prior reading at

540 nm with a photospectrometer Fluostar Omega. The calculation was done via linear regression fit.

4. Statistical analysis

Results were analyzed with the statistical software SPSS (SPSS Statistics 24) at a significance level of $P<0.05$. All experiments were carried out with at least three biological and three technical replicates. At first results were checked for normal distribution using Shapiro-Wilk-test. For variance homogeneity the Levene-test was used. When variance homogeneity was given an analysis of variance (ANOVA) was performed followed by the Student-Newman-Keuls test. Otherwise the Welch-ANOVA and the Tamhane-test was performed to determine differences among means. For the NR activity assay a Kruskal-Wallis-test followed by Dunn-Bonferroni-test was performed because no normal distribution and variance homogeneity was given.

III. Results

1. Selection of *nia1* homozygous line

Several seedlings of SALK_00416 line (original seeds from SALK) with a T-DNA insertion as shown in Error! Reference source not found. were grown on soil to facilitate self-pollination. After collection of seeds, new generation of plants was grown in pots. Subsequently, in order to select homozygous lines F2 plants were tested with a T-DNA- and *Nia1*-specific primer. **Figure 12** shows the results of positive selection of *nia1* plants. The presence of T-DNA at the expected position could be confirmed for line 1 and 3 using LBa1 and *Nia1*-specific primer. The use of the LBb1 primer led to the same result. Line 1 was finally used for further experiments.

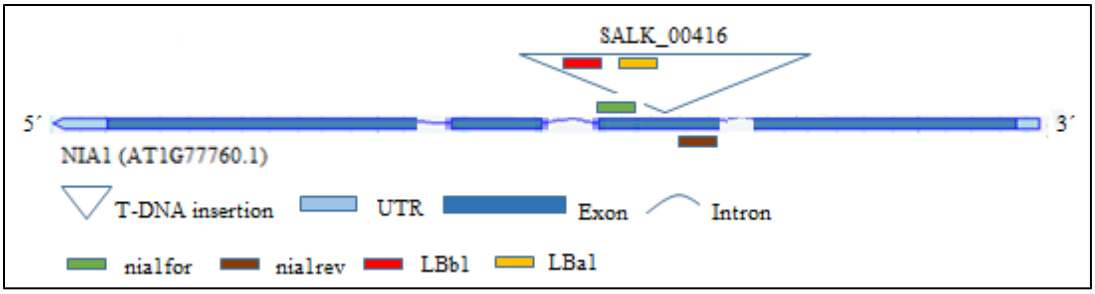

Figure 11 Intron-exon structure of *Nia1* gene and the position of T-DNA within the third exon in line SALK_00416. Primer combinations for selection of the homozygous line are shown.

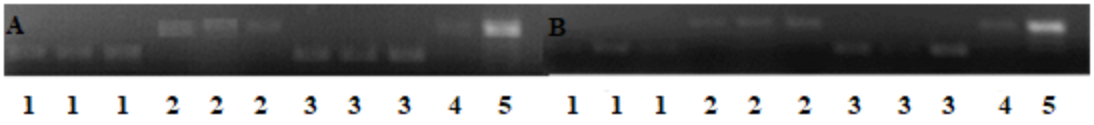

Figure 12 Selection of homozygous line of *nia1* mutant (SALK_00416). Used primer pair: nia1_Salk_004164with LBa1rev (A), PCR fragment size indicating homozygous line: 895 bp; and with LBb1rev (B), PCR fragment size for homozygous line: 696 bp. PCR fragment size indicating heterozygous line: 1186 bp. Numbers (1-4) indicate different tested mutant lines, 5 represents control plant.

2. Selection of *nia2* homozygous line

Several seedlings of SALK_088070 (original seeds from SALK) line with a T-DNA insertion as shown in **Figure 13** were grown on soil to facilitate self-pollination. After collection of seeds new generation of plants was grown in soil. Subsequently, in order to select homozygous lines F2 plants were tested with a T-DNA- and *Nia2*-specific primer. **Figure 14** shows the results of positive selection of *nia2* plants. The presence of T-DNA at the expected position could be confirmed for line 2 and 4 using LBb1 and the *Nia2*-specific primer. Line 2 was finally used for further experiments.

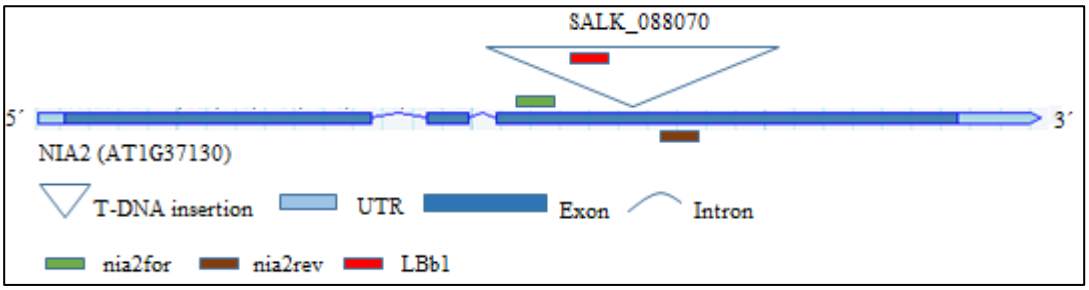

Figure 13 Intron-exon structure of *Nia2* gene and the position of T-DNA within the third exon in line SALK_088070. Primer combinations for selection of the homozygous line are shown.

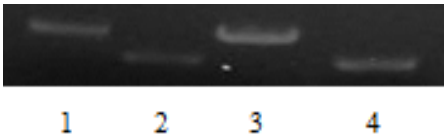

Figure 14 Selection of homozygous line of *nia2* mutant. Used primer pair nia2_Salk_008070 and LBb1, expected PCR fragment size indicating homozygous line: 670 bp. indicating heterozygous line: 802 bp. Numbers indicate different tested mutant lines.

3. Growth parameters

3.1. Fresh weight

The shoot fresh weight (FW) of all genotypes was not affected by fungal inoculation throughout all measured time points except in the case of Col-0 at 14 dpi, where inoculation with *S. indica* resulted in a significant increase in shoot FW. The mutant line *nia1* showed a similar trend, however the observed changes were not significant. It could be observed that both the un-colonized and colonized variant of *mpk6* exhibited the highest shoot FW throughout all time points. For root FW no significant influence could be observed for used genotypes at all measured time points. The results also showed that among all tested lines the *mpk6* had the highest root FW throughout all time points. All obtained results are shown in **Figure 15**.

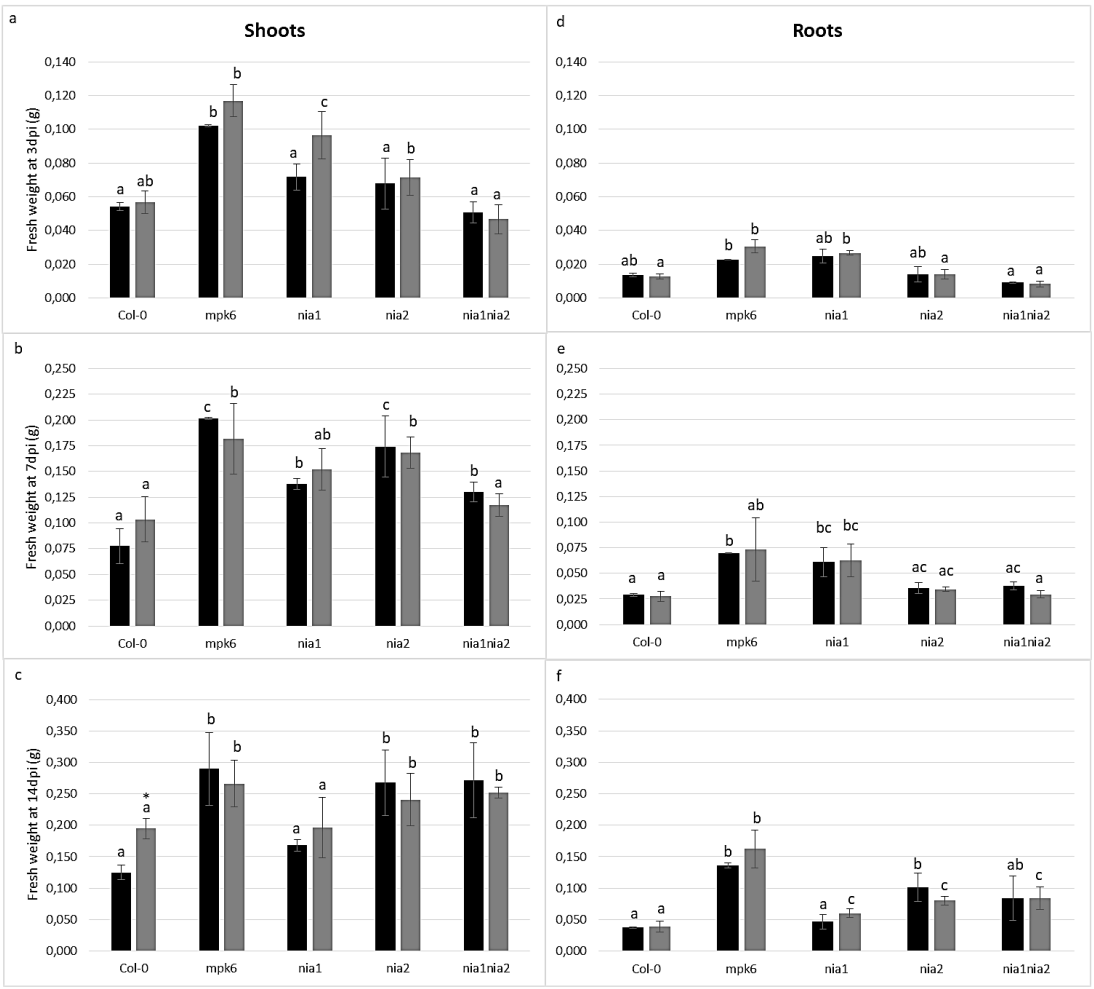

Figure 15 Dry weight of shoots (a-c) and roots (d-f) of *A. thaliana* colonized by *S. indica* at 3, 7 and 14 dpi. Black bar: control plants; grey bar: colonized plants. Values given are means ± SE of n=3. Each chart was extra calculated and included values were compared. Uniformly colored bars with different letters show significant differences between one variant and different genotypes. For simplicity, the comparison between non-colonized and inoculated plants was considered only within one genotype and marked with an asterisk (Univariate ANOVA followed by Tamhane-test, $P < 0.05$).

3.2. Dry weight

For all genotypes, no significant differences in dry weight (DW) at all measured time points between *S. indica* and non-colonized plants were observed except for *S. indica* colonized Col-0 plants, which showed a significant increase in shoot DW at 14 dpi. It was also observed that both the non-colonized and the colonized variant of *mpk6* exhibited the highest shoot DW throughout all tested time points. Also for root DW, no changes were detected upon fungal colonization. Both variants of *mpk6* had the highest root DW throughout all tested time points. All obtained results are shown in **Figure 16**.

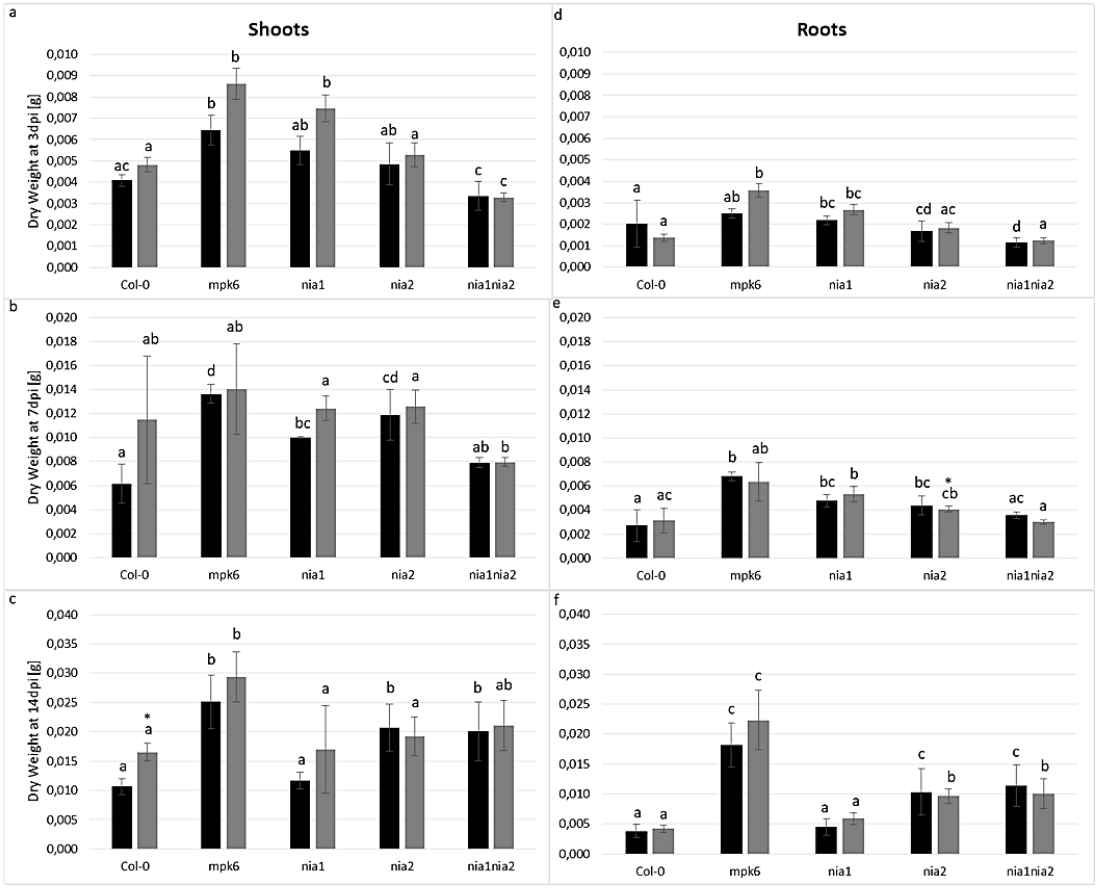

Figure 16 Dry weight of shoots (a-c) and roots (d-f) of *A. thaliana* colonized by *S. indica* at 3, 7 and 14 dpi. Black bar: control plants; grey bar: colonized plants. Values given are means ± SE of n=3. Each chart was extra calculated and included values were compared. Uniformly colored bars with different letters show significant differences between one variant and different genotypes. For simplicity, the comparison between non-colonized and inoculated plants was considered only within one genotype and marked with an asterisk (Univariate ANOVA followed by Tamhane-test, P < 0.05).

3.3. Root morphology

At all measured time points no increase in root length could be observed for all lines. In addition, all genotypes are shown to have a similar root length (see **Figure 18**).

In contrast, the results show significant impact of *S. indica* colonization on the number of root tips (see **Figure 17**). Among all lines Col-0 colonized by *S. indica* displayed a significant increase in root tip number at each tested time point. Additionally, a significant increase could be detected for *mpk6* at 3 dpi, for *nia1* at 3 and 14 dpi and *nia2* at 14 dpi. Non-colonized *mpk6* showed a comparably high number of root tips at 3, 7 and 14 dpi although there was no elevation in the root tip number triggered by *S. indica* colonization. Interestingly, the number of root tips of *nia1nia2* was not affected by fungal colonization. All obtained results are shown in **Figure 18**Figure 18.

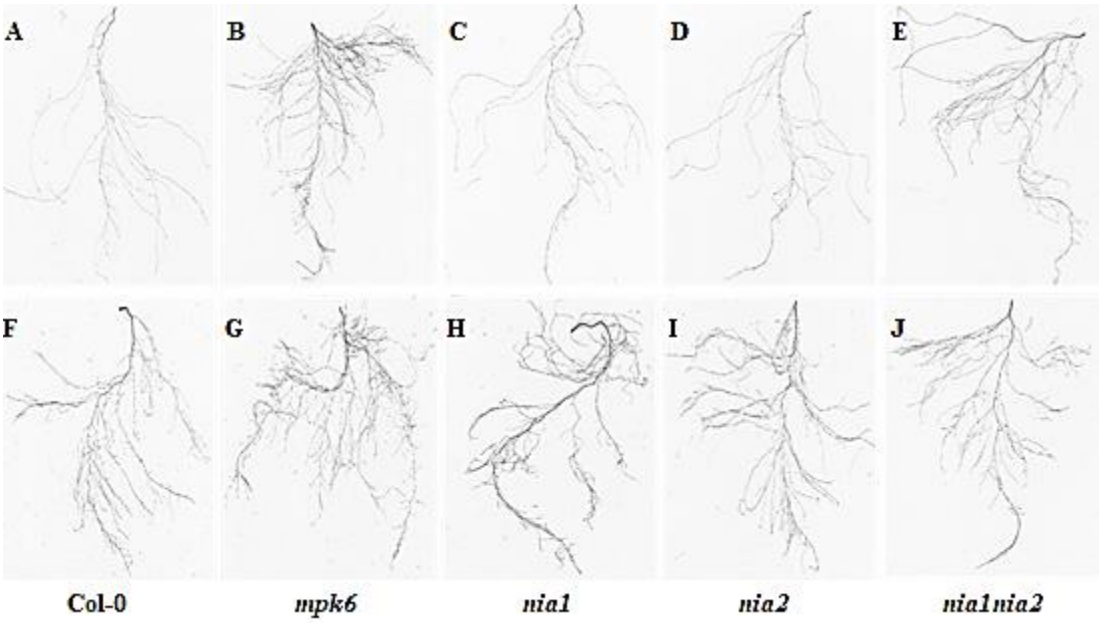

Figure 17 Impact of *S. indica* on *A. thaliana* roots 14 days after co-cultivation. A-E control; F-J colonized by *S. indica*.

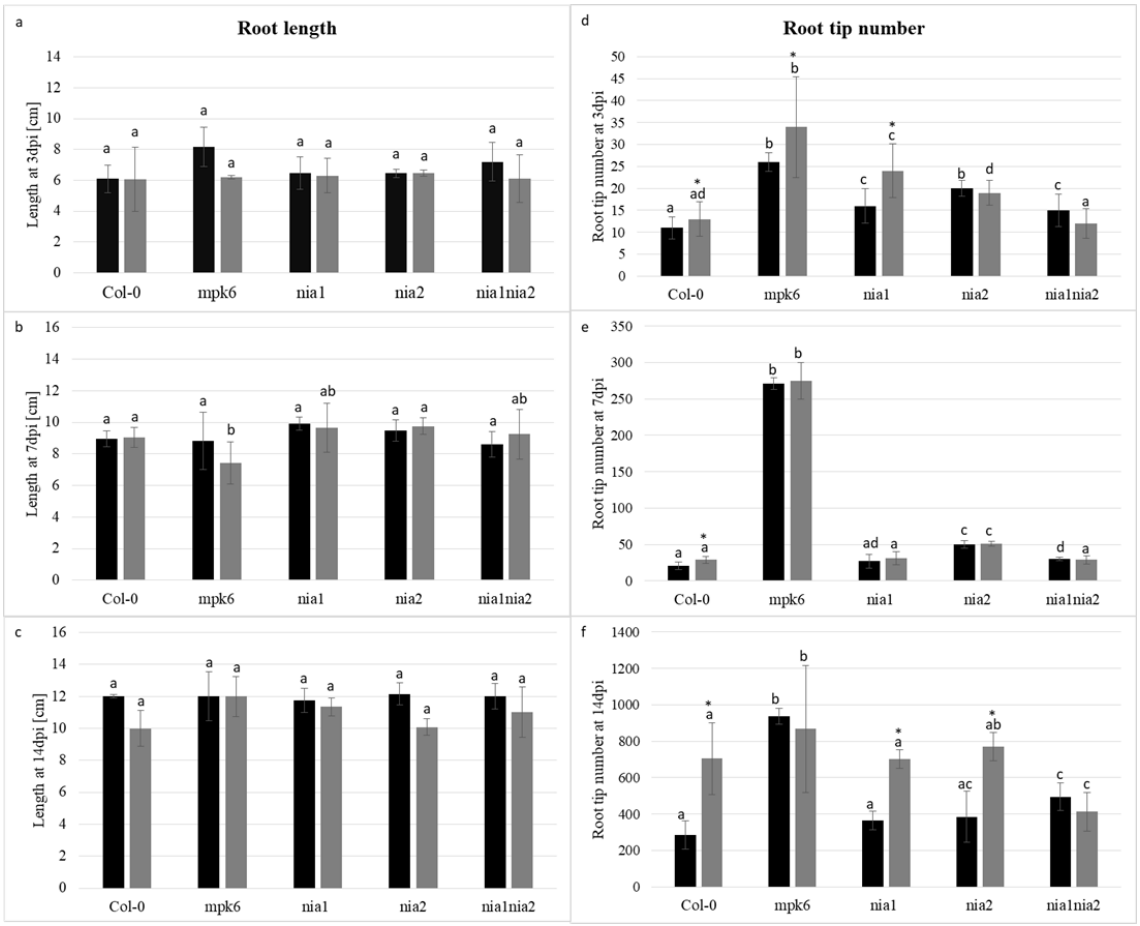

Figure 18 The effect of *S. indica* inoculation on (a,b,c) root length and number of (d,e,f) root tips in Col-0 and *nia1*, *nia2* and *nia1nia2* mutant lines at 3, 7 and 14 dpi. Black bar: control plants; grey bar: colonized plants. Values given are means ± SE of n=3 with six plants per replicate. Each chart was extra calculated and included values were compared. Uniformly colored bars with different letters show significant differences between one variant and different genotypes. For simplicity, the comparison between non-colonized and inoculated plants was considered only within one genotype and marked with an asterisk (Univariate ANOVA followed by Tamhane-test, P <0.05).

4. Colonization rate

A significantly lower colonization rate (CR) of *S. indica* was determined in *mpk6*, *nia1*, *nia2* and *nia1nia2* when compared to Col-0 at 3 dpi. Surprisingly, a significantly higher CR was detected for *nia1* at this time point. At 7 dpi a significantly lower CR was observed for *mpk6* and *nia2* roots in comparison with *nia1* and Col-0 but it was not significantly different from the CR estimated for *nia1nia2*. In contrast, no significant differences could be observed at 14 dpi. All genotypes showed a significant increase in CR over time, except for *nia1* and *nia1nia2*. Both mutant lines did not show any significant CR increase between 7 and 14 dpi. It should be noted that at 14 dpi Col-0 had the highest and *nia1nia2* the lowest CR. All obtained results are shown in **Figure 19**.

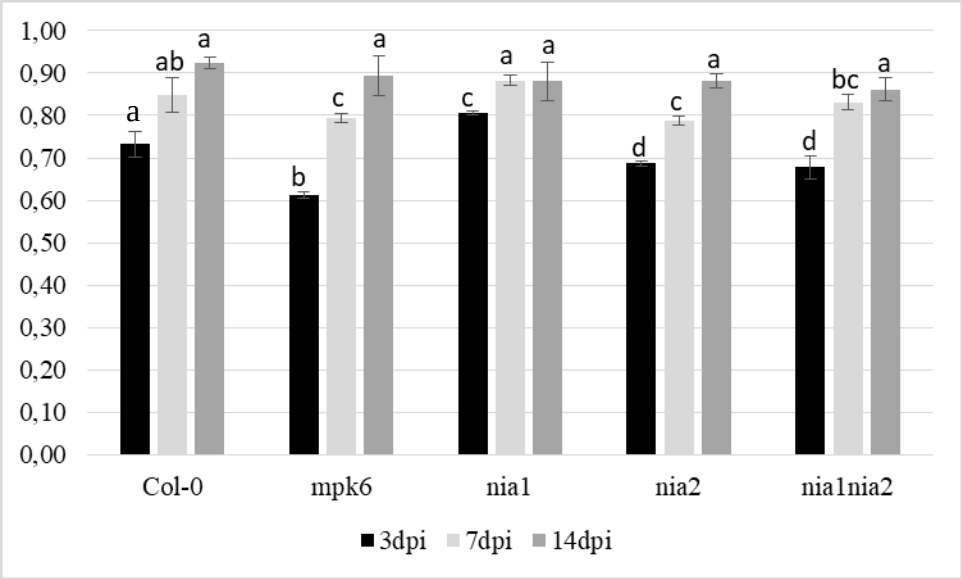

Figure 19 Relative colonization rate of *S. indica* in roots of Col-0 and *mpk6, nia1, nia2* and *nia1nia2* mutant lines at 3, 7 and 14 dpi. Different letters indicate significant differences between the genotypes at the same time point. (Student-Newman-Keuls-test, P <0.05).

5. Expression of *Nia1*

In shoots of plants colonized by *S. indica* significant changes in *Nia1* expression could be detected for *mpk6* and *nia2* in comparison to Col-0 at 3 dpi. At 3 dpi *Nia1* is up-regulated in Col-0 upon *S. indica* colonization. Furthermore, a down-regulation could be observed for Col-0 and *nia2* at 7 dpi and in Col-0 and *mpk6* at 14 dpi. In contrast, a strong up-regulation of *Nia1* was detected in *nia2* at 14 dpi.

In roots no differences of *Nia1* expression could be detected in all tested lines at all measured time points. The *S. indica* colonization did not influence the regulation of *Nia1* in all genotypes at all time points. The obtained results are shown in **Figure 20**.

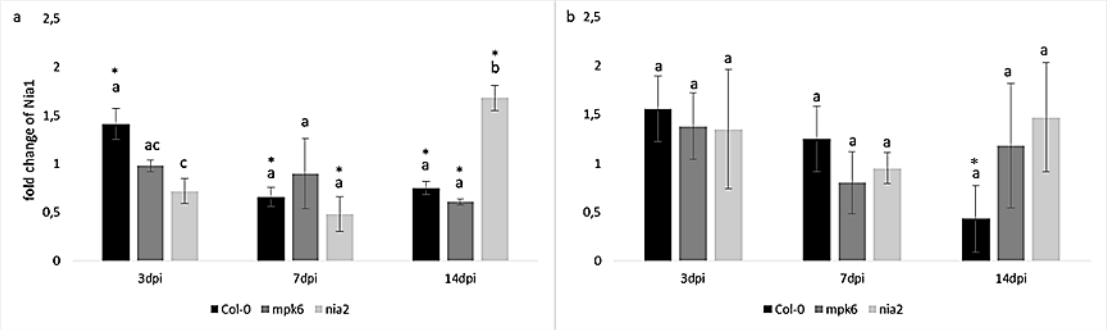

Figure 20 Relative expression of *Nia1* in shoots (a) and roots (b) of Col-0, *mpk6* and *nia2* at 3, 7 and 14 dpi. Asterisks indicate significant up- or down-regulation (One-Sample-T-test, tested against the value 1, *, P<0.05). Different letters indicate significant differences between genotypes at one time point. Values are means ± SE of n= 3 (Student-Newman-Keuls-test, P<0.05).

6. Expression of *Nia2*

Interestingly, an up-regulation at 3 dpi in shoots upon *S. indica* colonization could be only observed in *mpk6*. The shoots of all tested genotypes did not show changes in *Nia2* expression at 7 dpi. At 14 dpi a significant down-regulation of *Nia2* could be observed in Col-0 and *mpk6*. No significant differences in gene expression were detected between Col-0 and mutant lines.

Only at 3 dpi a significant difference in gene expression could be observed in *mpk6* and *nia1* roots compared with Col-0. An up-regulation of *Nia2* was detected in Col-0 and *mpk6* at 3 dpi. At 7 and 14 dpi the gene expression of *Nia2* did not changed upon *S. indica* colonization in Col-0 and *mpk6*. At 14 dpi a significant up-regulation of *Nia2* was detected in *nia1* roots. The obtained results are shown in **Figure 21**.

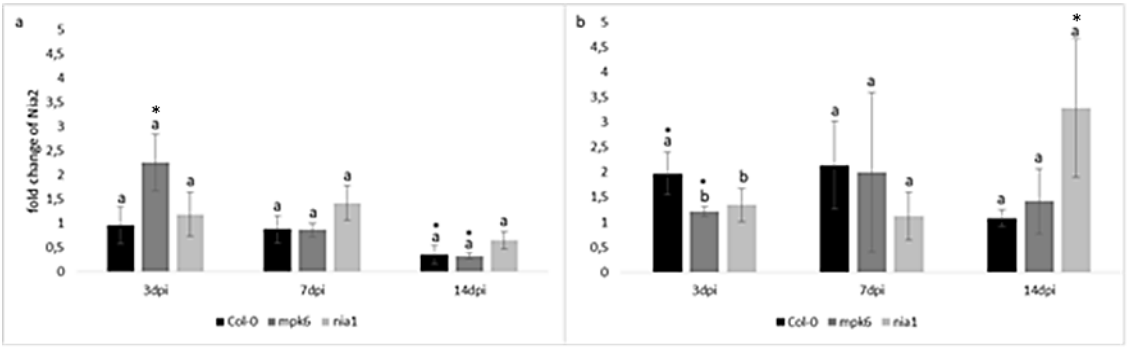

Figure 21 Relative expression of *Nia2* in shoots (a) and roots (b) of Col-0, *mpk6* and *nia1* at 3, 7 and 14 dpi. Asterisks indicate significant up- or down-regulation (One-Sample-T-test, tested against the value 1, *, P <0.05). Different letters indicate significant differences between genotypes at one time point. Values are means ± SE of n= 3 (Student-Newman-Keuls-test, P<0.05).

7. Nitrate reductase activity

Table 5 demonstrates that about 98% of total NRA could be obtained in shoots of Col-0 at all tested time points, whereas at 14 dpi no NRA was measured in roots anymore. A significant lower NR activity was observed in Col-0 shoots at 14 dpi in comparison to the other time points. Furthermore, for shoots and roots significant differences were detected at all measured time points. In both shoots and roots, NRA is decreasing with advancing plant age.

Table 5 Total nitrate reductase activity (NRA) in wild type shoots and roots at 3, 7 and 14 dpi. Different letters indicate significant differences between. Presented values are means of three replicates of four independent experiments ± SD (Kruskal-Wallis-test followed by Dunn- Bonferroni-test, P<0.05).

NR activity (µmol NO^{2-} * gFW^{-1}* h^{-1})			
Genotype	3 dpi	7dpi	14dpi
Col-0 shoot	4,34 ± 1,62[a]	2,43 ± 1,09[a]	1,18 ± 0,39[b]
Col-0 root	0,10 ± 0,09[c]	0,04 ± 0,03[c]	0,00 ± 0,00[c]

Table 6 Total nitrate reductase activity (NRA) in shoots of Col-0, *mpk6, nia1, nia2* and *nia1nia2* at 3, 7 and 14 dpi. Asterisks indicate significant differences in comparison to Col-0. Different letters indicate significant differences within one time point. Presented values are means of three replicates of four independent experiments ± SD (Kruskal-Wallis-test followed by Dunn- Bonferroni-test, P<0.05).

	NR activity (µmol NO^{2-} * gFW^{-1}* h^{-1})		
Genotype	3 dpi	7dpi	14dpi
Col-0	4,34 ± 1,62	2,43 ± 1,09	1,18 ± 0,39
Col-0 + *S. indica*	4,71 ± 2,57	3,72 ± 1,75	0,91 ± 0,21
mpk6	8,01 ± 3,82	6,69 ± 2,98	0,53 ± 0,30
mpk6 + S. indica	8,52 ± 2,99	4,11 ± 3,09	2,00 ± 1,77
nia1	3,79 ± 0,89	3,26 ± 0,24	0,83 ± 0,37
nia1 + S. indica	6,19 ± 1,31	4,29 ± 0,73	0,50 ± 0,18
nia2	0,21 ± 0,19	0,14 ± 0,04	0,11 ± 0,06
nia2 + S. indica	0,61 ± 0,45	0,69 ± 0,41	0,29 ± 0,05
nia1nia2	0,00 ± 0,00*	0,00 ± 0,00	0,00 ± 0,00
nia1nia2 + S. indica	0,00 ± 0,00*	0,00 ± 0,00	0,05 ± 0,05

In the present study total NRA was measured in shoots of Col-0, *mpk6*, *nia1*, *nia2* and *nia1nia2* at 3, 7 and 14 dpi (see **Table 6**). In all tested lines NRA is decreasing with advancing plant age irrespective of the colonization state. NRA of colonized plants did not differ significantly from un-colonized plants although Col-0, *nia1* and *nia2* colonized by *S. indica* showed an obviously higher NRA. At all tested time points NRA behaved similarly in Col-0 and *nia1* when comparing colonized plants with non-colonized plants, only at 14 dpi a significant lower NR activity was obtained for the variant *nia1 + S. indica*. Comparably high NRA was recorded for *mpk6* at 3 dpi. Low levels of NRA were detected in *nia2* shoots at 3 and 7 dpi. As expected no NRA could be measured in *nia1nia2* and differs significantly at 3 dpi in comparison to all other lines.

IV. Discussion

S. indica is an endophytic fungus that promotes plant growth and increases biotic and abiotic stress tolerance in *A. thaliana* (VARMA et al., 2013). In the draft genome of *S. indica* a gene encoding a nitrate transporter could not be identified and consequently this fungus grows slowly when fed with nitrate (LAHRMANN et al., 2013). However, probably to compensate this *S. indica* triggers up-regulation of *Nia2* in *A. thaliana* (SHERAMETI et al., 2005), which is the major gene coding for nitrate reductase (NR). It is the first enzyme in the nitrate assimilation pathway being responsible - among the others - for proper plant growth (WILKINSON and CRAWFORD, 1991). In addition MPK6 was shown to play an important role during plant-pathogen interaction (reviewed in TENA et al., 2011 and MENG and ZHANG; 2013) and to regulate NR (WANG et al., 2011). In this work plant growth, root morphology, colonization rate and NR activity were investigated at different time points corresponding to three subsequent colonization phases of *S. indica*: 3 dpi (colonization phase), 7 dpi (cell death-associated phase) and 14 dpi (fungal reproductive phase) (VARMA et al., 1999; JACOBS et al., 2011).

Plant biomass partitioning shifts to shoots when an increase in available nitrogen occurs (CAMBUI et al., 2011). Previous studies demonstrated that *S. indica* increases root and shoot weight in many plants, including *A. thaliana* (VARMA et al., 1999; PEŠKAN-BERGHÖFER et al., 2004; VAHABI et al., 2016). In this work the shoot fresh and dry weight of Col-0 was significantly higher upon *S. indica* colonization at 14 dpi when compared to un-colonized plants. Similarly, an increase in shoot fresh weight of *A. thaliana* seedlings 10 days after *S. indica* inoculation was reported (VADASSERY et al., 2009). Results from this work show that in *nia1* mutant shoot dry weight was increased at all time points but not significant upon *S. indica* colonization. Interestingly, a significant increase in shoot weight was obtained for *mpk6* shoots in comparison to Col-0 and *nia1nia2* at 3 dpi although *mpk6* plants are less colonized at this time point. It has been demonstrated that *A. thaliana* mutants impaired in salicylic acid- and glucosinolate-associated defense

are more susceptible to *S. indica* (SHERAMETI et al., 2008; JACOBS et al., 2011). MPK6 has been shown to be an essential part of these responses (XU et al., 2016; BECKERS et al., 2009). Thus, in plants lacking MPK6, which are more susceptible to *S. indica*, the early MPK6-related defense responses might be not triggered or triggered only partially. At early time point this can than result in faster root colonization and subsequent enhanced growth promotion. At later time points, however, the MPK6-dependent growth promotion seems to be not affected anymore. At all measured time points no influence of *S. indica* on shoot weight could be determined for *nia2* and *nia1nia2*. This proves that *Nia2* plays an important role in nitrite production and in shoot growth promoted by *S. indica.* Here, the root weight of Col-0, *nia1*, *nia2* and *nia1nia2* was not affected by *S. indica* and this is in agreement with the results of STREHMEL et al. (2016), who presented similar results using a hydroponic growing system. However, for roots, PEŠKAN-BERGHÖFER et al. (2004) showed that *A. thaliana* 5 days after co-cultivation with *S. indica* has a significant higher root biomass. In this work, also for *mpk6* no changes were determined at all measured time points. This is in line with previous result presented by VADASSERY et al. (2009), who demonstrated that 8 days after co-cultivation with *S. indica* no increase in root fresh weight in *mpk6* was observed. All collected results showing measurements on fresh and dry weight strongly indicate that shoot growth promotion triggered upon *S. indica* colonization in *A. thaliana* is only possible when NIA2 and MPK6 are fully functional.

Root architecture is greatly influenced by the availability and amount of nutrients in soil. Primary root length and root branching are essential prerequisites for plant survival in a special environment (THALER et al., 1998). In this work, no significant difference in root length between all colonized *S. indica* plants and control variants could be determined at all measured time points. Although upon *S. indica* colonization roots were shorter when compared to non-colonized plants. This is in line with a previous result demonstrated by STEIN et al. (2008), who showed that the main root length of 28-day-old *A. thaliana* plants is significantly reduced after 14 days of co-cultivation with *S. indica*. Only *mpk6* roots showed the same length irrespective of the

S. indica colonization at 14 dpi. This is not surprising since VADASSERY et al. (2009) found that an *mpk6* knock-out mutant failed to respond to growth promotion effects triggered by *S. indica*. It has to be stated that all genotypes used in this work exhibit the same primary root phenotype. Hence, it can be excluded that *Mpk6*, *Nia1* and *Nia2* could have an influence on the primary root length. At least for both genes coding NR, this is in agreement with ZHANG and FORTHE (1998), who using an *A. thaliana* double mutant lacking NRA, showed that the nitrate metabolism is not affecting the primary root length. In contrast, at 3 dpi a significant higher root tip number was detected for wild type, *mpk6* and *nia1*. The results suggest that during the early colonization phase, the *Nia2* gene mainly responses to *S. indica* colonization. These opposite results on primary root length (no changes) and root tip number (increase) strongly indicate that both are differently regulated in non-colonized plants as well as upon *S. indica* colonization. When not colonized, in comparison to other lines *mpk6* exhibited significantly higher number of root tips at all tested time points. However, upon *S. indica* colonization there is no difference in the number of root tips between control and colonized *mpk6*. This observation could be explained with the results presented by WANG et al. (2011), who demonstrated that MPK6 phosphorylates NIA2, which in turn increases production of nitric oxide (NO) being a repressor of primary and lateral root growth. The authors showed that *mpk6-2* and *mpk6-3* seedlings produce more and longer lateral roots than wild type plants did after application of the NO. Similarly, a significantly higher root tip number was observed for Col-0 at 14 dpi. Further, it is known that deficiency of *NIA1* and *NIA2* results in a significant reduction in NO synthesis (BRIGHT et al., 2006; MODOLO et al., 2006). Accordingly, at 14 dpi a significantly higher root tip number was observed for *nia1* and *nia2*. Why the number of root tips in line *nia1nia2* does not change upon *S. indica* colonization has to be analyzed in further experiments. A possible conclusion can be drawn from this work showing no increase in biomass, no increased production of lateral roots and a low colonization rate in *nia1nia2*. LEE et al. (2011) suggested that in Chinese cabbage the changes in root morphology and the increase in biomass are caused by efficient root colonization by *S. indica*. However, there are still other factors to consider, such as the significantly increased number of root tips in un-colonized

nia1nia2 compared to the wild type at all measured time points. Plants exhibiting well-developed root system show an enhanced ability to survive nutrient deficiencies (HODGE et al., 1999; COQUE and GALLAIS, 2006). This could be one of the reasons why *nia1nia2* can compensate for the lack of nitrate and absorb other nitrogen forms from the soil.

S. indica colonizes large range of hosts due to its ability to suppress the immune system of the plant (SCHÄFER et al., 2009). In *A. thaliana* this process is essential for its successful colonization and relies greatly on intact hormone signaling in which MPK6 seems to play a pivotal role (DANESHKHAH, unpublished results). Especially at the beginning of root colonization a number of MPK6- and hormone- related defense mechanisms against the fungus are active, e.g. higher expression of defense genes (SCHÄFER et al., 2009) and production of reactive oxygen species (NATH et al., 2016). At later colonization stages intracellular calcium concentration is increased in *A. thaliana* root cells that activates the mitogen-activated protein signaling pathways (VADASSERY et al., 2009). This work demonstrates, that at 3 dpi the lines *mpk6*, *nia2* and *nia1nia2* are colonized to a significantly lower extent when compared to the wild type. One explanation could be that in plants lacking MPK6 the fungus might struggle to affect the MPK6- and hormone-dependent defense responses. At early time point this can than result in lower root colonization. At later time points, however, the colonization as well as growth promotion seems to be not affected anymore. Another possible explanation delivers WANG et al. (2010) who suggested that MPK6 phosphorylates NIA2 generating NO, which affects the microtubular cytoskeleton (BLUME et al., 2013). This assumption is reinforced by KOHOUTOVA et al. (2015), who demonstrated that MPK6 is important for the regulation of the mitotic cytoskeleton and cell division processes, particularly under stress conditions. For AMF it was shown that after fungal recognition a pre-penetration apparatus in host cells is formed (3 dpi) by rearranging the cytoskeleton and the endoplasmic reticulum (GENRE et al., 2005). It is known for *S. indica* that it targets the cell volume by increasing the uptake of ions and water as well as rearranging the cytoskeleton in cells of Chinese cabbage (LEE et al. 2011). Although the formation of the pre-penetration apparatus was not shown during *S. indica* colonization, it could be possible that this

might be a reason why *S. indica* is not so effective in colonizing the roots of *mpk6* (lack of important cellular alterations). *S. indica* prefers developmentally 'older' tissue for colonization (DONG et al., 2013) as it was shown for barley where it mainly colonizes the root maturation zone consisting of root hairs rather than the meristematic and elongation zone proximal to the root tip (DESHMUKH et al., 2006). Similarly, in *A. thaliana* growth of *S. indica* is restricted to rhizodermal and cortical cells in maturation zone II, whereas meristematic and elongation zones are usually free of hyphae (JACOBS et al., 2011). Interestingly, it has been demonstrated that NO is involved in root hair formation and elongation (LOMBARDO et al., 2006), which together with results of this work suggests that root colonization by *S. indica* might depend on NO production or its function as signal molecule and further on the formation of root hairs. Surprisingly, at 3 dpi a very high colonization rate was observed in *nia1* roots perhaps due to its increased growth when compared to the wild type. In contrast, at 14 dpi the colonization rate remained nearly the same in both *nia1* and wild type, which might suggest the function of still unknown mechanism preventing the over-colonization of the roots. All genotypes showed a significant increase in CR over time, with the exception of *mpk6* and *nia2*. It might be indication that *Nia2* plays a decisive role in nitrogen supply and therefore no further fungal growth and root colonization occurred (14 dpi). A similar CR was obtained for the *nia1nia2* mutant exhibiting low NRA. This is supported by recent report showing that *S. indica* lacks genes involved in nitrogen metabolism (ZUCCHARO et al., 2011). This might be one of the reasons for the mutualistic symbiosis between *S. indica* and its host plants or a consequence of these relationships.

Plants upon co-cultivation with *S. indica* showed clear growth promotion, which is attributable to an increased nutrient supply. The results presented here showing an up-regulation of *Nia2* in colonized wild type roots at 3 dpi. This is in line with SHERAMETI et al. (2005) demonstrated that *S. indica* has a stimulatory effect on NR in *A. thaliana* roots. They also showed that the major NR gene *Nia2* is up-regulated by the fungus. In this work, at 14 dpi no further up-regulation could be detected, which can be attributed to limited amounts of nutrients and restricted space in Petri dishes. Interestingly, in the *Nia1*-deficient mutant an up-regulation of *Nia2* was detected

leading to the assumption that *Nia2* partly takes over *Nia1* function. This assumption is reinforced by the *Nia2*-deficient mutant, which exhibits a strong up-regulation of *Nia1* especially in the shoots, but it is also in the roots at 14 dpi. These findings are in line with the results presented by LOQUÉ et al. (2003), who showed elevated levels of *Nia1* in the roots of the G5 mutant (deletion in *Nia2*). Additional experiments demonstrated that the line *mpk6* showed a strong up-regulation of *Nia2* in shoots at 3 dpi. This might indicate a correlation between *Nia2* and growth enhancement, which could be observed in *mpk6* at 3 dpi. In this work, at 14 dpi *S. indica* colonization led to a significant reduction in *Nia1* expression in wild type but not in *nia2* and *mpk6* roots, respectively. A possible explanation could be that *Nia1* is up-regulated in *nia2* mutant to maintain the NRA and in *mpk6* due to the lack of its phosphorylation.

The enzyme NR plays the key role in the nitrate assimilation and is responsible for nitrite and NO production. In *A. thaliana* two genes, *Nia1* and *Nia2*, encode the NR. It is known that phosphorylation of NIA2 by MPK6 results in higher NRA (WANG et al., 2010). This could be confirmed in this work showing no significant NRA increase in *mpk6* mutants upon *S. indica* colonization at all tested time points. Similarly, significant differences were not observed in all other tested lines colonized by *S. indica* when compared to the respective un-colonized plants. However, a clear trend was visible showing higher NRA in colonized plants. For instance wild type, *nia1* and *nia2* showed an increased NRA upon *S. indica* colonization at 3 and 7 dpi. It seems that stimulation of NR by *S. indica* could be partially responsible for growth promotion. This indicates that the fungus is somehow able to control the NRA and that there is a putative involvement of MPK6 in this process. In several studies it has been demonstrated that in case of pathogen attack phosphorylation of NIA2 by MPK6 leads to an increased NRA and subsequently to different plant defense responses (WANG et al., 2011; MAGALHAES et al., 2000). However, in the *Mpk6*-deficient mutant the highest NRA was observed independent of the colonization state at 3 and 7 dpi. This suggests that MPK6 alone has no influence on nitrite production and the fact that *mpk6* had a significantly increased growth, especially at 3 and 7 dpi, reinforces this finding. Additionally, *mpk6* had the highest amount of lateral roots compared to the other lines. NRA was gradually decreasing in shoots and roots of all tested lines with advancing

plant age irrespective of the colonization state. This is in agreement with the results presented by CHEESEMAN et al. (2004), who showed that NRA in Col-1 shoots is declining to a low rate after 35 days. In this work, a total NRA of 98 % could be attained in Col-0 shoots. CHEESEMAN demonstrated that the shoots of Col-1 accounted about 90 % of the total NRA. Here, NRA measured in *nia1nia2* was at the detection limit and in *nia2* very low, but both were able to grow similar to the wild type. CHEESEMAN et al. (2004) suggests that plants will grow on every available N form in the rhizosphere.

The present study gives first indications how *S. indica* promotes plant growth and shows the involvement of MPK6 during this process. In further experiments it could be interesting to investigate the NO and the nitrite production contemporaneously. This would clarify to what extend *S. indica* promotes nitrite production, which in turn leads to an increased biomass. In addition, various methods measuring the NR activity should be used to compare results obtained in this work with those from other studies. This work shows that at 14 dpi (fungal reproduction phase) *nia2* and *nia1nia2* lines exhibited the lowest colonization rate compared with the wild type. It would be very interesting to examine the colonization stages using microscopic techniques to confirm these results.

VII. Summary

Serendipita indica, an endophytic fungus, is known to promote plant growth and it is suggested that this fungus increases the phosphate and nitrate uptake in plants. Maybe *S. indica* is a new tool for the improvement of plant production systems. The aim of this thesis was to provide new insights regarding the nitrate reductase and its role during the root colonization by *S. indica*. Since phosphorylation of NIA2 by MPK6 leads to an increase in NR activity and MPK6 affect the level of *S. indica* colonization this kinase was included into this work.

Within the scope of this work, different *A. thaliana* genotypes were used to investigate the influence of *Nia1*, *Nia2* and MPK6 on nitrate reductase activity, plant growth and their responses to *S. indica*. All experiments were carried out in petri dishes filled with Knop medium. For each measurement three time points, 3 dpi (colonization phase), 7 dpi (cell death-associated phase) and 14 dpi (fungal reproductive phase) were selected.

The results show that a significantly increased shoot weight was observed in the wild type colonized by *S. indica*. At 14 dpi a significantly increased number of root tips was detected in *nia1*, *nia2* and the wild type colonized by *S. indica*. There was no response of the double mutant *nia1nia2* to *S. indica*. An increased NR activity, however not significantly, could be detected in the wild type, *nia1* and *nia2* lines colonized by *S. indica* at 3 and 7 dpi. In comparison to the other lines *mpk6* failed to respond to *S. indica* despite increasing colonization over time.

The presented work provides first indications of the complex relationships between nitrate reductase, MPK6 and the colonization of an endophytic fungus. In order to expand the existing knowledge, further studies would be necessary.

VIII. Zusammenfassung

Serendipita indica, ein endophytischer Pilz, ist bekannt dafür das Pflanzenwachstum zu fördern. In der Literatur wird vorgeschlagen, dass dieser Pilz die Phosphat- und Nitrataufnahme in Pflanzen erhöht. Vielleicht ist *S. indica* ein neues Werkzeug zur Verbesserung der Anlagenproduktionssysteme. Daher war das Ziel dieser Arbeit, neue wissenschaftliche Erkenntnisse über das Enzym Nitratreduktase und *S. indica* zu liefern. Da MPK6 zur Erhöhung der Nitratreduktaseaktivität beiträgt und eine entscheidende Rolle bei der Pilzkolonisation spielt, wurde diese Kinase ebenfalls untersucht.

Im Rahmen dieser Arbeit wurden verschiedene *A. thaliana*-Genotypen verwendet um den Einfluss von *Nia1, Nia2* und MPK6 auf die Nitratreduktaseaktivität, das Pflanzenwachstum und ihre Reaktionen auf *S. indica* zu untersuchen. Das Experiment wurde in Petrischalen durchgeführt, die mit Knop-Medium gefüllt waren. Für jede Messung wurden drei Zeitpunkte: 3 dpi (Kolonisierungsphase), 7 dpi (Zelltod-assoziierte Phase) und 14 dpi (Pilz-Fortpflanzungsphase) ausgewählt.

Die Ergebnisse zeigen, dass ein signifikant erhöhtes Sprossgewicht im Wildtyp bei *S. indica* Kolonisation beobachtet wurde. Zum Zeitpunkt 14 wurde eine signifikant hohe Anzahl an Wurzelspitzen in den Mutanten *nia1, nia2* und dem Wildtyp, welche von *S. indica* kolonisiert wurden, nachgewiesen. Der Doppelmutanten *nia1nia2* zeigte keine Reaktionen auf eine *S. indica* Kolonisation. Eine erhöhte, aber nicht signifikant, Nitratreduktaseaktivität konnte in den mit *S. indica* kolonisierten Linien *nia1* und *nia2* sowie im Wildtyp bei 3 und 7 dpi gemessen werden. Im Vergleich zu den anderen Linien, zeigte der Mutant *mpk6* trotz der zunehmenden Kolonisation im Laufe der Zeit keine Reaktion auf die Kolonisierung durch S. *indica*.

Die vorliegende Arbeit liefert erste Hinweise auf die komplexen Zusammenhänge zwischen Nitratreduktase, MPK6 und der Kolonisation eines endophytischen Pilzes. Um das vorhandene Wissen zu erweitern, sind weitere Studien erforderlich.

IX. List of Abbreviations

A. thaliana	*Arabidopsis thaliana*
BSA	Bovine Serum Albumins
cDNA	Complementary DNA
ddH_2O	Double distilled water
DMSO	Dimethyl sulfoxide
Dpi	Days post inoculation
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
FAD	Flavin adenine dinucleotide
gDNA	Genomic Desoxyribonucleic acid
HCl	Hydrochloric Acid
KOH	Potassium Hydroxide
MPK6	Mitogen- activated protein kinase 6
mRNA	Messenger Ribonucleic acid
$Na_2HPO_4*12H_20$	Sodium dihydrogen phosphate dodecahydrate
$Na_2MoO_4 * 2H_20$	Dinatriummolybdat dihydrate
NADH	Nicotinamide adenine dinucleotide
$NaH_2PO_4*2H_2O$	Sodium dihydrogen phosphate dihydrate
$NaNO_2$	Sodium nitrite
$NaNO_3$	Sodium nitrate
NaOH	Sodium Hydroxide
NED	N-(1-Naphthyl) ethylene diamine dihydrochloride
NR	Nitrate reductase
NRA	Nitrate reductase activity
PCR	Polymerase chain reaction
PMSF	Phenylmethylsulfonyl fluoride

RIN	RNA Integrity Number
RNase	Ribonuclease
Rpm	Rounds per minute
RT- PCR	Real- time PCR
S. indica	*Serendipita indica*
SA	Sulfanilamide
Tab.	Table
T-DNA	Transfer DNA
Tris-HCl	Tris-(hydroxymethyl) aminomethan hydrochloride
UTR	Untranslated region

X. List of Figures

XI. List of Tables

XII. References

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XIII. Appendix

1. Kaefer medium composition for *S. indica*

Table 7 Kaefer medium (HILL and KÄFER 2001) composition for *S. indica* modified after JOHNSON et al., (2011)

Composition for 1 liter	Amounts
D-glucose	20.0 g
Peptone	2.0 g
Yeast extract	1.0 g
Casein hydrolysate	1.0 g
Macronutrient mix (20x)[1*]	50.0 ml
Micronutrient mix (100x)[2*]	10.0 ml
Fe-EDTA (100x)[3*]	1.0 ml
Agar (Daishin)	10.0 g
Adjust pH to 6.5 with 10N KOH Autoclave at 121°C for 20 min Add 1 ml filter sterilized vitamin mix (100x) 4* before pouring the media (temperature 45 °C to 50 °C) to petri dishes.	
1* macronutrient mix (20x) For 1 L: 12 g $NaNO_3$; 10.4 g KCl; 10.4 g $MgSO_4*7H_2O$; 30.4 g KH_2PO_4. All components are dissolved in sterile H_2O and then stored at 4 °C.	
2* micronutrient mix (100x) For 1 liter: 2.2 g $ZnSO_4*7H_2O$; 1.1 g H_3BO_3; 0.5 g $MnSO_4*4H_2O$; 0.16 g $CoCl_2*5H_2O$; 0.16 g $CuSO_4*5H_2O$; 0.11 g $(NH_4)6Mo_7O_{24}*4H_2O$. All components are dissolved in sterile H_2O and then stored at 4 °C.	
3* Fe-EDTA (100x) For 50 ml; 0.77 g Na_2EDTA; 0.556 g $FeSO_4*7H_2O$. All components are dissolved in sterile H_2O, heat to boil and stir for 30 min while cooling and then store at 4°C.	
4* vitamin mix (100x) For 100 ml: 10 g thiamin; 0.04 g glycerin; 0.01 g nicotinic acid; 0.01 g pyridoxine. All components are dissolved in sterile H_2O, then filter-sterilized and are stored as aliquots of 1 ml at -20°C.	

2. List of used chemicals

Table 8 List of used chemicals

Chemical	Name	Company	Origin
Tris-HCl	Tris-Hydrochloride	Carl Roth GmbH & Co.Kg	Germany
EDTA	Ethylenediaminetetraacetic acid	Carl Roth GmbH & Co.Kg	Germany
$Na_2MoO_4*2H_20$	Dinatriummolybdat dihydrate	Fluka Chemie GmbH	Switzerland
FAD	Flavin adenine dinucleotide	Sigma- Aldrich	Japan
DTT	Dithiothreitol	Invitrogen	Canada
BSA	Bovine Serum Albumins	Sigma- Aldrich	USA
PMSF	Phenylmethylsulfonyl fluoride	Sigma- Aldrich Chemie GmbH	Germany
$NaNO_3$	Sodium nitrate	E. Merck, Darmstadt	Germany
Na_2HPO_4* $12H_20$	Sodium dihydrogen phosphate dodecahydrate	E. Merck, Darmstadt	Germany
NaH_2PO_4* $2H_2O$	Sodium dihydrogen phosphate dihydrate	E. Merck, Darmstadt	Germany
β-NADH Grade 1	Nicotinamide adenine dinucleotide	Sigma- Aldrich Chemie GmbH	Germany
SA	Sulfanilamide	Sigma- Aldrich Chemie GmbH	China
NED	N-(1-Naphthyl) ethylene diamine dihydrochloride	Sigma- Aldrich Chemie GmbH	USA
$NaNO_2$	Sodium nitrite	Sigma- Aldrich Chemie GmbH	Germany
DMSO	Dimethyl sulfoxide	Sigma- Aldrich	France
HCl	Hydrochloric Acid	Sigma- Aldrich Chemie GmbH	Germany
NaOH	Sodium Hydroxide	Carl Roth GmbH & Co.Kg	Germany
Gamborg B5	Vitamin mixture	Duchefa Biochemie	Netherlands
Daichin Agar		Duchefa Biochemie	Netherlands
DanKlorix	Household bleach	Colgate Palmolive Ges.m.b.H.	Austria

Casein hydrolyzate		Fluka Chemie AG, Buchs	Switzerland
KCl	Potassium chloride	E. Merck, Darmstadt	Germany
$MgSO_4*7H_2O$	Magnesium sulphate heptahydrate	Sigma- Aldrich Chemie GmbH	Germany
KH_2PO_4	Potassium dihydrogen phosphate	AppliChem GmbH	Germany
$ZnSO_4*7H_2O$	Zinc sulphate heptahydrate	Carl Roth GmbH & Co.Kg	Germany
$CuSO_4*5H_2O$	Copper(II) sulfate pentahydrate	Carl Roth GmbH & Co.Kg	Germany
$MnSO_4*4H_2O$	Manganese(II) sulphate tetrahydrate	Sigma- Aldrich Chemie GmbH	Germany
$(NH_4)6Mo_7O_{24}*4H_2O$	Ammonium molybdate tetrahydrate	Sigma- Aldrich Chemie GmbH	Germany
$CoCl_2*5H_2O$	Cobalt(II) chloride hexahydrate	Sigma- Aldrich Chemie GmbH	Germany
H_3BO_3	Boric Acid	E. Merck, Darmstadt	Germany
$FeSO_4*7H_2O$	Iron(II) sulphate heptahydrate	E. Merck, Darmstadt	Germany
$Na_2EDTA*2H_2O$	Disodium ethylenediaminetetraacetate dihydrate	Duchefa, Haarlem	Netherlands
Nicotinic acid		Fluka Chemie AG, Buchs	Switzerland
Pyridoxine		Sigma- Aldrich Chemie GmbH	Germany
Glycine		Carl Roth GmbH & Co.Kg	Germany